Herpes simplex viruses type 1 and 2 photoinactivated in the presence of methylene blue transform human and mouse cells \textit{in vitro}

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Received February 21, 2017; revised March 29, 2017; accepted June 26, 2017

Summary. – Three strains of herpes simplex virus, K17\textsuperscript{syn-} and HSZP\textsuperscript{syn+} of type 1 (HSV-1) and US\textsuperscript{syn-} of type 2 (HSV-2), were photoinactivated in the presence of methylene blue and used to infect 3 cell lines, normal human lung tissue cells (MRC-5), mouse epithelial cells (NIH3T3), and human lung carcinoma cells (A549). The virus titer and phenotype of cells were evaluated to compare the characteristics of normal and carcinoma cells infected with non-syncytial (non-syn) and syncytial (syn) strains of herpes simplex viruses. We found that the cells of both normal cell lines infected with photoinactivated K17\textsuperscript{syn-} and US\textsuperscript{syn-} but not HSZP\textsuperscript{syn+} acquired transformed phenotype accompanied by the presence of virus. Surprisingly, the infection with photoinactivated viruses K17\textsuperscript{syn-} and US\textsuperscript{syn-} but not HSZP\textsuperscript{syn+} resulted in the suppression of the transformed phenotype of A549 cells. Using nested PCR, herpesviral DNA was identified in newly transformed cells and cells that lost the transformed phenotype. The effect of putative herpesvirus-related growth factors (HRGF) produced by cells infected with photoinactivated viruses was quantified and compared. Since methylene blue is currently used in phototherapy of herpetic lesions, these results raise the question of whether such therapy is risky to human health.

Keywords: herpes simplex viruses; photoinactivation of viruses; methylene blue; putative herpesvirus-related growth factors; transformed phenotype; phototherapy

Introduction

At present, there is no direct evidence that human alphaherpesviruses herpes simplex virus type 1 (HSV-1) or 2 (HSV-2) may be oncogenic. Although some seroepidemiological and biological studies have pointed to the possible role of HSV-2 in the development of cervical cancer (Nachmas \textit{et al}., 1974; Goldberg and Gravell, 1976; Rapp and Red, 1976; Thankamani \textit{et al}., 1985), other attempts have failed to support this idea (Vonka \textit{et al}., 1984; Lehtinen \textit{et al}., 2002). Both HSV-1 and HSV-2, however, are still considered to be in relationship to some cancers such as epithelial skin cancer (Ypiranga and de Morales, 2009), squamous cell carcinoma (Metgut \textit{et al}., 2012; Gupta and Metgud, 2013), oral cancer (Shillitoe and Silverman, 1979; Stele and Shillitoe, 1991), oropharyngeal cancer (Starr \textit{et al}., 2001), thyroid cancer (Stamatiou \textit{et al}., 2016), and prostate cancer (Thomas \textit{et al}., 2011, Yun \textit{et al}., 2016). Attempts to demonstrate the ability of human alphaherpesviruses to transform cells \textit{in vivo} failed as their transforming potential is masked by extensive cytopathology and the death of infected cells. The elimination of cell death could be accomplished for example by UV irradiation. UV-irradiated HSV-1 and HSV-2 lost their ability to cause cytopathic effect (CPE) and foci of transformed cells appeared in the culture monolayer (Duff and Rapp,
1971; Rapp and Duff, 1973). Recently, similar results were obtained by Mrázová et al. (2015) with UV-irradiated murine gammaherpesvirus 68 (MHV-68).

Another approach to eliminate the lytic activity of viruses is photodynamic inactivation, when a virus is treated with photosensitizer such as neutral red (NR) or methylene blue (MB) and exposed to visible light. This treatment results in the destruction of virus infectivity (Yen and Simon, 1978; Schnipper et al., 1980; Bodylak et al., 1983; Costa, 2012). Studies employing the photoinactivation of HSV-1 and HSV-2 in the presence of NR have shown that under conditions, in which virus infectivity is destroyed, the photoactivated virus can morphologically transform the cells in vitro, similar to UV-irradiated virus (Li et al., 1975; Kucera et al., 1977; Rapp and Kemeny, 1977). All cells transformed by HSV-1 and HSV-2 shared similar properties. They synthetized HSV-specific antigens detected by immunofluorescence, contained virus DNA, were resistant to superinfection with homologous transforming virus, and produced tumors in weanling animals after subcutaneous inoculation. Tumor-bearing animals produced neutralizing antibodies against transforming virus (Rapp and Duff, 1973; Rapp and Reed, 1976; Kucera et al., 1977). Similar results were obtained in cells transformed by UV-inactivated MHV-68. Virus DNA and virus antigen could be detected by PCR and immunofluorescence methods; furthermore, the disappearance of actin bundles was observed, indicating that transformed cells might be oncogenic (Mrázová et al., 2015).

In the 1990s, a novel class of compounds resembling growth factors, which cannot be related to any hitherto known cellular products, was found produced by herpesviruses and characterized. These putative HRGF have the ability to transform normal non-transformed cells and to suppress the transformed phenotype when added to transformed cells. Both activities of putative growth factors could be neutralized not only by antisera to the corresponding virus, but also by some monoclonal antibodies directed against viral gB glycoproteins (Golais et al., 1988, 1992a,b; Konvalina et al., 2002; Šupolíková et al., 2015). It has been also found that non-syncytial but not syncytial strains of HSV-1 were able to produce these compounds during infection of cells (Golais et al., 1992a,b). To take a position on this, we included into the study HSZP strain of HSV-1, suggested to be associated with the absence of HRGF in some cells. HSZP is a non-pathogenic syncytial strain of HSV-1 forming large polykaryocytes in infected cell cultures due to a syn+ mutation in the C-terminal endodomain of glycoprotein B (Rajčáni et al., 1996,1999; Küdelová et al., 1998). Several important in vitro and molecular studies have shown abolished shutoff function of the HSZP virus due to at least four out of six specific mutations seen in vhs polypeptide encoded by the UL41 gene (Matis and Küdelová, 2001). In vivo studies have shown that HSZP is poorly reactivable and shows limited neural spread in mice (Kúdelová et al., 1996).

As already mentioned, photoactivated herpesviruses may transform cells in vitro, thus indicating that they might be oncogenic in vivo. A human clinical therapeutic procedure based on photodynamic inactivation has been developed. This treatment consists of applying a photosensitizing dye to herpetic lesions and then exposing them to visible light. Taking into account previous data, this procedure can represent a potential cancer risk (Bockstahler et al., 1979; Rapp and Li, 1982). NR was one of the first, although not very effective, photosensitizers used for treatment of herpetic lesions (Myers et al., 1975, 1976; Roome et al., 1975). However, satisfactory results have been obtained using MB (Chang et al., 1975; Tardivo et al., 2005, 2012; Marotti et al., 2009, 2010; Sperandio et al., 2009; Ramalho et al., 2015). No transforming ability of any strain of HSV-1 or HSV-2 photoinactivated in the presence of MB has been demonstrated as yet.

The aim of this study was to evaluate the impact of in vitro infection with photoinactivated HSV-1 and HSV-2 of normal non-transformed and transformed cells, respectively, contributing to the clarification of the possible health hazard of the aforementioned treatment of herpetic lesions. Some issues of HSV-1- and HSV-2-associated putative growth factors were also addressed.

Materials and Methods

Viruses. The HSV-1 K17 strain was obtained from the MRC-University of Glasgow Centre for Virus Research, Glasgow (Scotland, UK). This strain has non-syncytial (in this study designed as HSV-1 K17\textsuperscript{non-syn}) plaque morphology (Shubak-Sharpe, 1973). The HSV-1 HSZP strain and the HSV-2 US strain were obtained from the collection of the Department of Molecular Pathogenesis of Viruses, Institute of Virology, Biomedical Research Center, Bratislava (Slovakia). HSZP is a non-pathogenic syncytial (in this study designed as HSV-1 HSZP\textsuperscript{non-syn}) strain of HSV-1 forming large polykaryocytes in infected cells. HSV-2 US has non-syncytial (in this study designed as HSV-2 US\textsuperscript{non-syn}) plaque morphology.

Cells. The following cells were used: BHK-21 cells (ATCC CCL-10) derived from the kidneys of 1-day-old hamsters (Stoker and MacPherson, 1964); MRC-5 (ATCC CCL-171), a human diploid cell culture established from the normal lung tissue of a 14-week-old male fetus (Jacobs et al., 1970); NIH3T3 cells, mouse fibroblasts obtained from Swiss albino mouse embryo tissue (Todaro and Green, 1963); and A549 cells (ATCC CRM-CCL-185), a human lung carcinoma cell line originating from an explant culture of carcinomatous lung tissue from a 58-year-old Caucasian male (Giard et al., 1973). The cells were cultivated in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 7% fetal bovine serum (FBS), 1% L-glutamine, and 1% Penicillin/Streptomycin/
Amphotericin B (PSA) and incubated at 37°C in a humidified 5% CO₂ atmosphere.

Photodynamic inactivation. Methylene blue (Loba Feinchemie GmbH, Fischamend, Austria) diluted in phosphate-buffered saline was added to 1 ml of virus suspension of HSV-1 K17™, HSV-1 HSZP™, and HSV-2 US™ containing 1 x 10⁶ PFU in 6 cm plastic Petri dishes to a final concentration of 10⁻⁴ mol/l. The virus suspension was then exposed to light-emitting diodes (LED) at a distance of 5 cm at 37°C. A monochromatic LED red light system (homemade device, λmax = 650 nm, 16.7 Wm⁻²) was used for the experiments. The emission spectrum of the light system was measured with an optical fiber high resolution Red Tide USB-650 fiber optic spectrometer (Ocean Optics, Dunedin, FL, USA). Virus suspension samples taken at intervals 0, 30, 60, 90, and 120 s after exposure to visible light were used to infect BHK-21 cells and the virus titer of each individual sample was evaluated. Appropriate photoinactivated samples of viruses were diluted 10 and 100 times and then used to infect triplicates of monolayers of MRC-5, NIH3T3, and A549 cells grown in 24-well plates. Infected cells were observed daily for the appearance of morphological changes. Virus suspensions added to the cells containing the same MB concentration but not exposed to visible light (kept in the dark) and the cells infected with the same dose of non-photoinactivated virus served as negative and positive controls, respectively.

Titration of viruses by plaque assay. Ten-fold dilutions of virus samples in the amount of 0.1 ml were inoculated onto monolayers of BHK-21 cells in the 24-well plates in triplicate. After an incubation period of 60 min to allow the virus to adsorb to the cells, the monolayers were overlaid with a nutrient medium containing 0.5% methylcellulose that prevented virus release from the infected cells so that only cell-to-cell spread occurred. After 72 h, the medium containing methylcellulose was removed and the cells were fixed with 4% formalin and stained with carbolfuchsin. The plaques were counted and the virus titers were expressed as PFU/ml. Data were expressed as mean ± SD obtained from triplicates.

Morphological and cytological examination of cells. The cells cultivated in 6-cm plastic Petri dishes were fixed with 50% methanol for 10 min, then 10 min with 100% methanol, and stained using the Giemsa-Romanowski method.

Titration of HRGF. To evaluate the transforming activity of HRGF, 10-fold dilutions (from 10⁻⁶ to 10⁰) of media from cells infected with photoactivated viruses were added to NIH3T3 or MRC-5 cells grown in 96-well microplates in quadruplicates. The same media samples were added to carcinoma A549 cells and the transformed phenotype-suppressing activity of HRGF was evaluated and compared to the controls. We found that the titer of all photoactivated viruses continuously decreased with increasing light exposure time. As shown in Fig. 1, exposure of 30 s to visible light in the presence of MB caused about 30% decrease of infectivity in all viruses, while the strongest effect on HSV-2 US™ could be observed. Furthermore, the infectivity of all viruses was around 50% after 120 s of exposure.

Following 7 days of culture of NIH3T3 and MRC-5 infected with photoactivated HSV-1 K17™, HSV-1 HSZP™, and HSV-2 US™, most of the cells of both cell lines showed incomplete CPE (about 70–90%), whereas cells infected with untreated viruses were destroyed by a total CPE within 3 days. After 7 days of culture, a relatively small portion of cells of both cell lines infected with any of the photoactivated HSV-1 K17™ and HSV-2 US™ remained undestroyed by virus infection but acquired altered morphology resembling transformation with a multilayered “criss-cross” pattern of growth. Moreover, cells could be subsequently passaged without the loss of transformed phenotype. We found that NIH3T3...
Fig. 1
Reduction of infectivity of HSV-1 K17syn−, HSV-1 HSZP<sup>mon+</sup>, and HSV-2 US<sup>syn−</sup> exposed to visible light for 30–120 s
Untreated viruses served as negative controls. Data are expressed as mean ± SD obtained from triplicates.

Fig. 2
Changes in NIH3T3 cells phenotype caused by infection with photoinactivated HSV-1 K17<sup>syn−</sup> (a and b) and HSV-1 HSZP<sup>mon+</sup> (c) (a) and (b) cells after the fifth and tenth passage; (c) cells after the fifth passage; (d) uninfected cells (negative control); magnification 50x.

Fig. 3
Changes in MRC-5 cells phenotype caused by infection with photoinactivated HSV-2 US<sup>syn−</sup>.
(a) cells after the fifth passage; (b) uninfected cells (negative control). Magnification 50x.

MRC-5 cells displayed the transformed phenotype caused by the photoinactivated HSV-1 K17<sup>syn−</sup> (data not shown) and HSV-2 US<sup>syn−</sup> (Fig. 3a). We found that both non-syn herpes simplex viruses of both types used in this study induced stable transformed phenotype in MRC-5 as well as NIH3T3 cells. On the other hand, the infection with photoinactivated syncytial HSV-1 HSZP<sup>mon+</sup> failed to induce such transformation and the phenotype of NIH3T3 (Fig. 2c) and MRC-5 cells (data not shown) remained the same as that of the cells infected with untreated virus or uninfected cells (Figs. 2d and 3b).

In the following experiments, we investigated virus presence in both of cell lines transformed by photoinactivated...
herpes simplex viruses by nested PCR method. Despite of
the different effect observed on cells after infection with
photoinactivated non-syn (HSV-1 K17\textsuperscript{syn} and HSV-2 US\textsuperscript{syn}) and
HSV-1 HSZP\textsuperscript{syn} strains, we identified the presence of
viral DNA in MRC-5 cells (Fig. 5a) and NIH3T3 cells (data
not shown), respectively.

Next, in experiments on carcinoma A549 cells infected with
photoinactivated viruses, we observed an opposite effect found
for normal cells: a change of transformed phenotype into phe-
notype resembling the phenotype of normal non-transformed
cells. The cells infected with HSV-1 K17\textsuperscript{syn} (Fig. 4a) and HSV-2
US\textsuperscript{syn} (data not shown) retained a normal, non-transformed
phenotype after at least 5 following passages. On the other
hand, photoinactivated HSV-1 HSZP\textsuperscript{syn}, unlike non-syn herpes
simplex viruses, failed to induce such a change in the trans-
formed phenotype of A549 cells, which remained unchanged
after at least 6 following passages (Fig. 4b). The investigation
by molecular methods of A549 cell lines, which after infection
with photoinactivated viruses lost transformed phenotype or
remained transformed, has shown the presence of DNA of
HSV-1 K17\textsuperscript{syn} and HSV-1 HSZP\textsuperscript{syn} in relevant cells (Fig. 5b).

The fact that photoinactivated syncytial HSV-1 HSZP\textsuperscript{syn}
did not induce the morphological changes either in non-
transformed NIH3T3 and MRC-5 cells or transformed A549
cells suggest that cells of all these cell lines infected with this
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In this study, we demonstrated the acquisition of the
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In this study, we demonstrated the acquisition of the
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as the change of the transformed phenotype of A549 cells
towards the normal one following infection with photoinactivated non-syncytial herpes simplex viruses HSV-1 K17<sup>syn-</sup> and HSV-2 US<sup>syn-</sup>. Similar changes of phenotype in normal and transformed cells were recently described for NIH3T3 and HeLa cells infected with UV-irradiated MHV-68 and suggested to be related to putative growth factor MHGF-68 (Mrázová et al., 2015; Šupolíková et al. 2015).

Finally, we compared the production of virus-related growth factors by all cell phenotypes involved. As described in Material and Methods, we quantified the effect of HRGF induced in NIH3T3, MRC-5, and A549 cells by infection with all 3 photoinactivated viruses. Data obtained confirmed that HRFG displayed the strongest effect on the phenotype of NIH3T3 cells infected with photoinactivated non-syncytial strains of HSV-1 and HSV-2. However, no effect of photoinactivated HSV-1 HSZP<sup>syn+</sup> on the phenotype of any cell line was observed (Fig. 6).

Results suggest that the non-syncytial phenotype of herpes simplex virus strains is required for the production of HRGF, which plays an important role in the change of the phenotype of virus-infected cells. It is interesting that the effect of HRGF induced by infection of normal mouse NIH3T3 cells, but also human carcinoma A549 cells, with non-syncytial HSV-2 is stronger than that caused by non-syncytial HSV-1. This work represents the first study on the absence of effects of photoinactivated HSV-1 HSZP<sup>syn+</sup> on normal as well as transformed cells what is most likely related to the absence of putative growth factors.

As follows from this study, the infection of non-transformed cells with HSV-1 or HSV-2 photoinactivated in the presence of MB may result in their transformation, which might render this method hazardous. The aim of this work was not to doubt the success of the phototherapy on herpetic lesions. The data gained here pose the question of whether the virus is capable of transforming the cells in vivo and could also be oncogenic in an organism. Nevertheless, the development of modern photodynamic therapy procedures as well as the use of suitable photosensitizers could minimize or remove the possible side effects of this treatment, as suggested by Wainwright (2003).

Acknowledgements. This work was supported by grant #APVV-0621-12 of the Slovak Research and Development Agency and grant VEGA#2/0087/17. The authors appreciate the HSV-1 BAC kindly provided by Prof. Ulrich H. Koszinowski.

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