Transformation of Cells by Photoinactivated Murine Gamma-Herpesvirus 68 during Nonproductive and Quiescent Infection

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Keywords

Murine herpesvirus 68 · Photoinactivation · Cell transformation · Quiescent infection

Abstract

Infection of human MRC-5 cells and mouse NIH-3T3 cells with a murine gamma-herpesvirus (MuHV-4 strain 68; MHV-68) photoinactivated by visible light in the presence of methylene blue (MB) resulted in nonproductive infection and the appearance of morphologically transformed cells. Two stably transformed cell lines were derived from both of these cell types and were confirmed to contain both viral DNA and antigen. Next, a quiescent MHV-68 infection in MRC-5 and NIH-3T3 cells was established after cultivation at 41 °C in the presence of phosphonoacetic acid. Following the exposure of quiescently infected cells to visible light for 120 s (5 times daily for 6 days) in the presence of MB, both MRC-5 and NIH-3T3 cells were observed to acquire transformed phenotypes. The cytopathic effect was observed in cells after 4–5 passages, after which the cells degenerated. However, when human interferon (IFN)- α and mouse IFN- β were added to the media of quiescently infected MRC-5 and NIH-3T3 cells during the photoinactivating procedure, 2 stable transformed cell lines

containing both viral DNA and the antigen were obtained and resembled those attained following nonproductive infection with photoinactivated virus.

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Introduction

Photodynamic therapy (PDT) using light-sensitive compounds (photosensitizers), such as neutral red (NR), methylene blue (MB), proflavine, and others [1], which become toxic to targeted bacteria, fungi, or cancer cells upon exposure to visible light, has recently found wide application [2, 3]. Similarly, viruses treated with photosensitizers can be rendered sensitive to visible light [4–6].

Photoinactivation of herpesviruses was intensively studied in the 1970s and the 1980s. Yen and Simon [7] observed that herpes simplex virus type 1 (HSV-1) became sensitive to light when treated with NR, and similar results were obtained by Schnipper et al. [8] and Badylak et al. [9] with pseudorabies virus, using MB, light, and electricity. Müller-Breitkreuz and Mohr [10] observed that MB and light treatment of herpesviruses gives rise to DNA damage and blocks virus replication. Kelleher and

Varani [11] demonstrated that NR caused reduction in the reactivation rate of HSV-2 from quiescent infection. Recently, Latief et al. [12] demonstrated that PDT is effective in eliminating acyclovir-resistant as well as nonresistant strains of HSV-1 from infected cells in vitro without harmful effects on host cells. On the other hand, attempts to use photoinactivation for the treatment of herpetic lesions have given contradictory results. Felber et al. [13] were successful in curing recurrent HSV infection using several heterocyclic dyes and fluorescent light. In contrast, Roome et al. [14] concluded that photoinactivation with NR was not effective in the treatment of acute genital herpes infections. Similarly, Meyers et al. [15, 16] failed to demonstrate the photodynamic inactivation of recurrent herpetic infection using NR. Recently, Marotti et al. [17, 18] were successful in the use of PDT as a treatment for herpes labialis, and similar results were obtained by Tardivo et al. [19] and Ramalho et al. [20]. However, some studies have demonstrated that photoinactivation of HSV-1 and HSV-2 in the presence of NR resulted in transformation of the cells in vitro, and thus PDT may be potentially oncogenic [21–23]. Wainwright [4] believes that the modern approach to phototherapy as well as the use of new, thoroughly tested photosensitizers could help eliminate potential side effects such as the transformation of cells. Recently, transformation of MRC-5 and NIH-3T3 cells in vitro was demonstrated after infection with UV-irradiated alpha-herpesviruses, HSV-1 and HSV-2 [24–26]. Moreover, UV-irradiated murine gamma-herpesvirus 68 (MHV-68) enabled the transformation of human and mouse dermal fibroblast cells in which the disappearance of actin bundles was demonstrated [27].

In this study, for the first time, infection with MHV-68 photoinactivated by visible light in the presence of MB was demonstrated. MHV-68 – a natural pathogen of murid rodents – is a member of the Gammaherpesvirinae subfamily. This subfamily also includes the Epstein-Barr virus (EBV) and Kaposi sarcoma-associated herpesvirus (HHV-8) that infect humans and are oncogenic. Because MHV-68 is similar to both EBV and HHV-8, in that infected mice develop lymphoproliferative disease and display high-grade lymphomas a long time after in vivo infection, it is currently used as an animal model for the study of human gamma-herpesvirus pathogenesis and oncogenesis [28–31]. Unlike EBV and HHV-8, MHV-68 replicates in various cultured cells, reaching high titers, and displaying cytopathic effect (CPE) and plaques in the agar overlay assay, thus having a greater resemblance to alpha-herpesviruses than gamma-herpesviruses [29, 32,

33]. Here, we studied the effect of photoinactivation on cells infected with treated MHV-68 and on cells quiescently infected with nontreated virus. We evaluated the transformation of human embryonic lung cells (MRC-5) and mouse fibroblast cells (NIH-3T3): (i) following infection with MHV-68 photoinactivated by exposure to visible light in the presence of MB, and (ii) by photoinactivation induced in cells quiescently infected with MHV-68, thereby modifying the experiments performed by Kelleher and Varani [11] and by Colberg-Poley et al. [34].

Materials and Methods

Virus

We used MHV-68 clone f2.6 originated from the isolate of MHV-68 kindly provided by Prof. Mistríková (Comenius University, Bratislava, Slovakia) that was plaque-purified twice, as described previously [35]. The virus was propagated and titrated using baby hamster kidney fibroblast (BHK-21) cells derived from the kidneys of 1-day-old hamsters (ATCC CCL-10), as described previously [36].

Cells

The following cells were used: MRC-5 cells (ATCC CCL-171), a human diploid cell culture established from the normal lung tissue of a 14-week-old male fetus; NIH-3T3 cells, mouse fibroblasts obtained from Swiss albino mouse embryo tissue. The cells were cultivated in DMEM (Dulbecco's modified Eagle's medium) supplemented with 7% fetal bovine serum, 1% L-glutamine, and 1% prostate-specific antigen, and incubated at 37°C in a humidified 5% CO₂ atmosphere.

Photoinactivation of MHV-68 and the Infection of Cells

MB (Loba Feinchemie GmbH, Austria) diluted in phosphate-buffered saline was added to 1 mL of MHV-68 suspensions containing 1×10^6 PFU in 6-cm plastic Petri dishes to obtain a final concentration of 0.1 nmol/L. Virus suspensions were then exposed to LED light at a distance of 5 cm at 37°C. A monochromatic LED red light system (homemade device, $\lambda_{\text{max}} = 650 \text{ nm}$, 15.0 Jcm^{-2}) was used for the experiments. The emission spectrum of the light system was measured at a high resolution of the Red Tide USB650 Fiber Optic Spectrometer (Ocean Optics, USA). The samples of photoinactivated virus taken at 30-s intervals from 30 to 120 s were used to infect BHK-21 cells. After 1 day of culture, the titer of photoinactivated virus in the culture media was evaluated as previously described [37] and compared to the controls. The plaques were counted and the virus titers were expressed as PFU/mL. Data obtained from the plaque assays performed in triplicate were expressed as the mean $\pm$ SD. Appropriate samples of photoinactivated MHV-68 were diluted 10 times and used to infect 24-h-old monolayers of MRC-5 or NIH-3T3 cells grown in 96-well plates. Infected cells were observed daily for the appearance of CPE or morphological changes and later passaged. Untreated virus diluted in the same manner served as a negative control. Virus suspensions added to cells containing the same MB concentrations but kept in the dark served as an additional control.

Fig. 1. The reduction of infectivity in BHK-21 cells of MHV-68 photoinactivated by exposure to visible light at different time intervals in the presence of MB evaluated by plaque assay. Data are expressed as the mean $\pm$ SD from experiments performed in triplicate. K, control BHK cells containing MB but infected with non-photoinactivated MHV-68 (kept in the dark).

Morphological and Cytological Examination of Cells

Cells cultivated in 6-cm plastic Petri dishes were fixed with 50% methanol for 10 min, followed by 100% methanol for 10 min, and stained using the Giemsa-Romanowsky method.

Establishment of Quiescent Infection of Cells with Untreated MHV-68 and Photoinactivation

MRC-5 and NIH-3T3 cells were grown in 24-well plates for 48 h until a confluent cell monolayer was formed. The cultivating medium was then replaced by that containing PAA (an inhibitor of DNA synthesis; Sigma) at a concentration of 200 μ g/mL and the cells were cultured at 41°C. Following 24 h of cultivation, cells were infected with MHV-68 at a multiplicity of infection of 0.01 PFU/mL and cultivated for the next 24 h at 41°C in the presence of PAA. Thereafter, the medium was replaced with a medium without PAA and with 2% inactivated fetal bovine serum and 0.1 nmol/L of MB. The cells were then cultivated at a temperature shifted down to 37°C. Before the photoinactivation of MRC-5 and NIH-3T3 cells quiescently infected with MHV-68, human leucocyte interferon (IFN)- α and mouse IFN- β (PBL Interferon Source) were added to obtain a final concentration 200 U/mL. Next, the cells were exposed to LED light for 120 s 5 times daily (at 2-h intervals) for 6 days, whereas they remained intact during the night. Cells were then trypsinized and thereafter passaged using the DMEM medium without MB and IFN. MRC-5 and NIH-3T3 cells cultured in the presence of relevant IFNs in the medium without MB and those cultured without IFNs and MB in the medium served as controls.

Polymerase Chain Reaction

DNA from transformed cell lines was isolated and screened for the presence of MHV-68 DNA by standard nested polymerase chain reaction (PCR) targeting the ORF 50 gene of MHV-68, as previously described by Kúdelová et al. [37]. The sequences of forward and reverse primers amplifying a 382-bp-long nested PCR product were as follows: ORF50/F1: 5'-AACTGGAACTCTCTGTGGC-3', ORF50/R1: 5'-GGCCGCAGACATTTAATGAC-3',

and ORF50/F2: 5'-CCCCAATGGTTCATAAGTGG-3', ORF50/R2: 5'-ATCAGCACGCCATCAACATC-3'. DNA of MHV-68 BAC served as the positive control. All of the PCR work performed complied with generally known strict protocols to cross-contamination controls, such as pipetting the template in a separated PCR box and room, and using a PCR mixture without a template as a negative control. The nested PCR products were resolved on 1.5% agarose gels and samples yielding PCR products of the expected size were evaluated.

Immunofluorescence

Indirect immunofluorescence assay was performed. Coverslip cultures of MRC-5 and NIH-3T3 cells were fixed in 4% paraformaldehyde and stained with rabbit hyperimmune anti-MHV-68 serum and Protein G Alexa Fluor 488 (Invitrogen). Nuclei were labeled with DAPI (Invitrogen).

Results

Infection of MRC-5 and NIH-3T3 Cells with Photoinactivated MHV-68

To evaluate the effect of photoinactivation on MHV-68, we exposed virus samples of a titer of approximately 1×10^6 PFU/mL to visible light in the presence of MB. The infectivity of MHV-68 photoinactivated at various time intervals from 30 to 120 s was determined using BHK-21 cells. We observed that the titer of photoinactivated virus continuously decreased with increasing light exposure time. As shown in Figure 1, an exposure of 30 s caused an approximate 20% decrease in virus infectivity, while it was reduced to approximately 10% after 120 s of photoinactivation. When MRC-5 and NIH-3T3 cells were infected with virus photoinactivated for 90 and 120 s and cultivated as shown in Figure 2, the morphology of both cell types changed, resembling a transformed phenotype. We observed that MRC-5 as well as NIH-3T3 cells maintained such morphological changes for the next 4–5 passages (approx. 30 days) and then entered crisis. They ceased to form a confluent monolayer and detached from the bottom. However, after additional cultivation (10–15 days), a few foci of morphologically transformed cells with a marked “crisscross pattern of growth” characteristic appeared in approximately 20% of the NIH-3T3 cells (Fig. 3a) as well as in the MRC-5 cells (data not shown). These cells remained transformed throughout the following passages and allowed us to derive a novel stable transformed cell line from NIH-3T3 (Fig. 3b) as well as MRC-5 cells (data not shown). Both cell lines were then evaluated for the presence of viral antigen and DNA. MHV-68 antigen was clearly visible throughout the cytoplasm of the transformed cells of both cell lines. Transformation of

Fig. 2. Passing of MRC-5 and NIH-3T3 cells infected with photoactivated MHV-68.

Fig. 3. Transformed NIH-3T3 cells derived from cells infected with photoactivated MHV-68. Cells after crisis (a), transformed cells after the 3rd passage (b), and control NIH-3T3 cells (c). Original magnification, $\times 50$.

Fig. 4. Transformed MRC-5 cells derived from cells infected with photoactivated MHV-68 stained with rabbit hyperimmune anti-MHV-68 serum and Protein G Alexa Fluor 488, detected by the immunofluorescence method. Transformed cells after the 3rd passage (a) and control MRC-5 cells (b). Original magnification, $\times 50$.

Fig. 5. PCR detection of MHV-68 DNA in transformed MRC-5 and NIH-3T3 cells. DNA extracted from cells derived from cells infected with photoinactivated MHV-68. Lanes 1–3: MRC-5 cells after the 3rd, 7th, and 10th passages; lanes 4–5: NIH-3T3 cells after the 3rd and 8th passages. DNA extracted from cells derived from quiescently infected MHV-68 cells photoinactivated in the presence of human α -IFN. Lanes 6–7: NIH-3T3 and MRC-5 cells after

the 7th passage; lane 8: noninfected MRC-5 cells; lane 9: 100-bp ladder (Fermentas); lane 10: MHV-68 BAC DNA (nested PCR; positive control); lane 11: MHV-68 BAC DNA (1-step PCR with nested primers; positive control); lane 12: no template (nested PCR; negative control); lane 13: no template (1-step PCR with nested primers; negative control).

Fig. 6. Transformed MRC-5 cells derived from cells quiescently infected with MHV-68 that were photoinactivated in the presence of human leucocyte IFN- α . Transformed cells after the 5th passage (**a**) and control MRC-5 cells (**b**). Original magnification, $\times 50$.

MRC-5 cells after the 3rd passage (Fig. 4a) and of NIH-3T3 cells after the 4th passage (data not shown) induced by infection with photoinactivated MHV-68 was observed to be associated with visible disintegration of actin filaments and the presence of virus antigens, as detected by immunofluorescence analysis. Using PCR, the presence of MHV-68 DNA was detected in transformed MRC-5 cells after the 3rd, 7th, and 10th passages (Fig. 5, lanes 1–3), and in transformed NIH-3T3 cells after and the 3rd and 8th passages (Fig. 5, lanes 4–5).

Photoinactivation of Cells Quiescently Infected with MHV-68

In the following experiments, we investigated the effect of photoinactivation on cells that were “latently” or quiescently infected with nontreated MHV-68. We established a quiescent infection of MRC-5 and NIH-3T3 cells with MHV-68 following a shift in incubation tempera-

ture of cultures from 41 to 37°C and PAA removal, and demonstrated that no CPE appeared and no infectious virus was detected during the subsequent 4- to 5-day period of culture (data not shown). The CPE subsequently appeared after 6 days of culture when the cells had degenerated. However, when MB was added to the medium of quiescently infected cells and they were exposed to visible light, morphological changes appeared after 5 days of culture resembling those of the cells infected with photoinactivated virus (as shown in Fig. 3b). However, it was not possible to passage these cells more than 4–5 times before they died. When human IFN- α and mouse IFN- β was added to the medium of quiescently infected MRC-5 and NIH-3T3 cells before the photoinactivation procedure, morphological changes in cells resembling the transformed phenotype were much more pronounced compared to those in the cells cultured without IFN. Cells of both types could be subsequently passaged without the

appearance of CPE and infectious virus in the medium. Subsequent serial passaging resulted in stable transformed MRC-5 cells (Fig. 6a) and NIH-3T3 cells (data not shown), respectively. We demonstrated that none of the IFNs had an effect on the morphology of quiescently infected cells untreated by photoinactivation. Finally, we confirmed by PCR that, similar to the cells obtained after infection with photoinactivated virus, both NIH-3T3 and MRC-5 cells contained MHV-68 DNA after the 7th passage (Fig. 4, lanes 6 and 7).

Discussion

In the 1970s, photoinactivation following exposure to NR and MB was reported to be effective in the treatment of recurrent herpes simplex infections in humans [13, 38]. Thereafter, several works pointed at a transforming, and thus potentially oncogenic potential of herpes simplex viruses photoinactivated in the presence of NR [22, 23, 38]. However, it was demonstrated in some cases that NR was not effective in the treatment of herpetic lesions [14–16]. In our previous study, we demonstrated that UV-inactivated MHV-68 can transform cells in vitro [27], thus resembling the transforming ability of several UV-inactivated herpesviruses belonging to the subfamily Alphaherpesvirinae, such as HSV1, HSV2, and EHV [24–26, 39]. Recent studies have reported that normal cell lines such as MRC-5 and NIH-3T3 (used in this study) cultured in the presence of MB acquired a transformed phenotype accompanied by the presence of virus when they were infected with photoinactivated nonsyn strains (K17 and Kupka) but not syn strains (HSZP) of HSV-1 [40].

No photoinactivation experiments on gamma-herpesvirus using MB and visible light have been hitherto performed. In this work, we demonstrated that the infectivity of photoinactivated MHV-68 in BHK-21 cells strongly decreased; however, the virus remained infectious after a 120-s-long exposure to visible light in the presence of MB. We confirmed that infection of MRC-5 and NIH-3T3 cells with photoinactivated MHV-68 was nonproductive as no infectious virus was produced by these cells. In cells that overcame crisis and could be subsequently passaged, MHV-68 DNA and virus antigen were detected and such a nonproductive infection resulted in the appearance of a morphologically transformed phenotype in cells. Two stable transformed cell lines of human and mouse cell origin were derived and demonstrated to contain both the viral DNA and the antigen. We demonstrat-

ed that the transformed MRC-5 and NIH-3T3 cell lines remained stable until the 10th and the 8th passage, respectively. We observed that treatment of MHV-68 infection with MB exposed to visible light can result in non-productive infection and the transformation of cells. The transforming ability of photoinactivated MHV-68 seems to be similar to that of MHV-68 exposed to UV light [27]. Thus, MHV-68 appears to be the first gamma-herpesvirus for which photoinactivation has been demonstrated to cause the acquisition of a transforming phenotype by infected cells.

In subsequent experiments, we focused on the effect of photoinactivation on latent infection typically occurring after the clearance of acute infection with herpesviruses, including MHV-68, in the host. To date, no reliable in vitro model exists to investigate the molecular mechanisms involved in controlling herpesvirus latency or virus reactivation during recurrent infection caused by herpesviruses. Few systems have been developed in which herpesvirus is retained in a nonreplicating state known as quiescence. Quiescent infection of cell cultures may provide an important and amenable means of studying the molecular mechanisms that mimic herpesvirus latency in vivo. Such in vitro models can be established at supraoptimal temperatures [41–43] or in the presence of inhibitors of DNA synthesis [34]. Here, we established quiescent infection of MHV-68 in MRC-5 and NIH-3T3 cells by infection with a low dose of virus (0.01 PFU/mL), cultivation in the presence of PAA, and a temperature shift from the usual 37 to 41 °C. Next, cells were photoinactivated by a procedure previously described, enabling the induction of transformation of the cells infected with treated virus. First, we observed that photoinactivated MRC-5 and NIH-3T3 cells quiescently infected with non-treated virus acquired the transformed phenotype. However, the CPE was observed relatively early, after 4–5 passages, before the cells degenerated. Next, when human IFN- α and mouse IFN- β were added to the media of quiescently infected cells during the photoinactivating procedure, we obtained cells which demonstrated a transformed phenotype associated with visible disintegration of actin filaments. The morphology of cells of both types resembled the morphology of those obtained after non-productive infection with photoinactivated MHV-68. Based on knowledge that IFN suppresses the reactivation of quiescent herpesvirus we used IFN to activate/accelerate the transformation of infected cells. However, the mechanism of this action is at present not fully understood. This phenomenon must be studied in future in more detail. Moreover, 2 stable transformed cell lines

were established in which the presence of both viral DNA and the antigen was confirmed. We demonstrated that both transformed cell lines derived from photoinactivated cells quiescently infected with untreated MHV-68 remained stable until the 7th passage. Notably, nonphotoinactivated, quiescently infected cells (used as a control) cultured in the presence of IFNs did not demonstrate changes in their morphology. The only difference observed was the prolonged duration of quiescent infection, wherein CPE in cells was observed approximately 48–72 h later (following the removal of PAA and temperature shift).

Taken together, our results demonstrate that we prepared transformed MRC-5 and NIH-3T3 cell lines derived from the corresponding normal cell lines infected with photoinactivated MHV-68 as well as from photoinactivated MHV-68-infected quiescent cells. We demonstrated that photodynamic treatment of MHV-68 may lead, under some circumstances, to the transformation of

cells that are nonproductively or latently infected with the virus. Although we cannot conclusively claim that PDT of recurrent herpes may be oncogenic, considering the results of this study on gamma-herpesvirus-infected cells, the possibility should be ruled out or confirmed in animal models of herpetic latency.

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Disclosure Statement

The authors declare that they have no conflicts of interest.

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