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Short communication

Up-regulation of Murid herpesvirus 4 ORF50 by hypoxia: Possible implication for virus reactivation from latency

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Abstract

Murid herpesvirus 4 (MuHV-4) is a member of the Gammaherpesvirus subfamily capable to establish a long-lasting latency and induce occasional malignancies. Because MuHV-4 is associated with cancer in a subset of virus-infected mice and because tumor development is often linked with hypoxia, we studied the influence of hypoxia on the biology of this virus. Using immunofluorescence and FACS analysis we detected increased proportion of MuHV-4 positive cells in the latently infected NB-78 cell line exposed to low oxygen conditions compared to normoxic controls. Moreover, the expression of ORF50, a crucial gene responsible for switch from latency to lytic virus replication, was induced upon the exposure of NB-78 cells to hypoxia. Luciferase reporter assays with ORF50 promoter confirmed the hypoxia-dependent induction. Transient co-transfections with hypoxia inducible factors showed that HIF-2α is a more potent activator of ORF50 expression than HIF-1α. Our results confirm that the MuHV-4 life cycle is influenced by low oxygen concentration.

Keywords: Murid herpesvirus 4; Hypoxia; Latent infection; ORF50

Murid herpesvirus 4 (MuHV-4), also known as Murine gammaherpesvirus 68 (MHV-68), is a member of the Gammaherpesvirus subfamily (Gammaherpesvirinae) together with Kaposi's sarcoma-associated herpesvirus (KSHV) and Epstein-Barr virus (EBV). These viruses are able to establish life-long latent infection and are often associated with various types of malignancies, such as Kaposi's sarcoma and B-cell lymphomas (Hsu and Glaser, 2000; Sunil-Chandra et al., 1992b; Viejo-Borbolla and Schulz, 2003). Beside the prototypic MHV-68 strain, several MuHV-4 isolates were identified (Blaskovic et al., 1980; Kozuch et al., 1993; Virgin et al., 1997). These isolates are closely related, but differ in some aspects of in vivo infection. In general, following intranasal inoculation of mice, MuHV-4 replication initially occurs in the lungs and virus subsequently infects multiple organs and establishes a latent or persistent infection in the spleen (Rajcani and Kudelova, 2005; Sunil-Chandra et al., 1992a,b; Weck et al., 1996). At late times after the initial infection, approximately 10% of mice develop lymphoproliferative disease characterized by lymphomas associated predominantly with spleen and mesenteric lymph nodes (Mistrikova et al., 2006; Sunil-Chandra et al., 1994).

In contrast to human retroviruses, which are found in tumors integrated into the cellular DNA, gammaherpesviruses generally persist in cells in the form of episomes tethered to the cellular chromatin by viral nuclear protein. During tumor development, oncogenic viruses are exposed to dramatic micro-environmental changes. One of the most profound phenomena of tumor growth is hypoxia as a result of insufficient oxygen supply of quickly proliferating tumor cells (Giatromanolaki and Harris, 2001). The primary response to the lack of oxygen at the molecular level is the stabilization of the α subunit of HIF-1 transcriptional complex, a key regulator of the genes involved in the adaptation to the hypoxic stress (Semenza, 2000). HIF-1 transcriptional complex activates transcription of a variety of cellular genes that contain HIF-1 binding site(s), so called hypoxia response ele-

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ment (HRE, 5′-RCGTG-3′) in their regulatory regions (Wang and Semenza, 1993)).

Virus–hypoxia interactions were initially studied in connection with oncolytic viruses utilized for gene therapies or as shuttle vectors to carry various cytotoxic genes (Binley et al., 2003; Hardcastle et al., 2007; Liu et al., 2007). A variety of different DNA and RNA viruses have been tested as oncolytic agents or powerful delivery vectors potentially useful for treatment of tumors with extensive hypoxic areas, which are more resistant to conventional cancer therapies (Alain et al., 2002; Andreansky et al., 1996; Balachandran et al., 2001; Binley et al., 2003; Sinkovics and Horvath, 2000). Influence of hypoxia on viral biology became an attractive topic in the effort to find the most effective strategies to combat cancer cells. It was recently found that hypoxia can induce lytic replication of KSHV via direct binding of HIF to the functional hypoxia response element within the KSHV genome (Cai et al., 2006; Davis et al., 2001; Haque et al., 2003). In addition, hypoxia increases expression of the viral pro-angiogenic factor vIL-6. These results suggest that hypoxia-induced KSHV activation may be important for the life cycle of the virus, but may also contribute to the viral pathogenesis by releasing several human pro-angiogenic factors with the impact on the proliferation of peripheral vascular endothelial cells (Bais et al., 1998; Masood et al., 1997). Hypoxic induction of EBV lytic cycle as a result of increased level of viral Zta protein was also described (Jiang et al., 2006). Based on complex studies, it is clear that the relationship between hypoxia and virus is rather mutual: the expression of several viral genes can be influenced by hypoxia and vice versa, the expression of oxygen-dependent master regulator HIF-1α can be affected by several viral genes (Lopez-Ocejo et al., 2000; Tang et al., 2007; Yoo et al., 2003, 2004).

MuHV-4 has been lately widely studied and became accepted as a good animal model to study human members of the gammaherpesvirinae subfamily, where in vivo models are not available (Simas and Efstathiou, 1998). MuHV-4-established latent infection in B-lymphocytes may result in development of lymphomas in the spleen and other organs (Mistrikova et al., 2006; Sunil-Chandra et al., 1994).

The fact that MuHV-4 could be exposed in vivo to stimuli arising from tumor micro-environment, and that hypoxia is one of the most profound hallmarks of tumor development, prompted us to study interaction between MuHV-4 and hypoxia in the NB-78 cell culture model. Recently established NB-78 cell line was derived from a cervical lymphoma of a mouse infected with MuHV-4 isolate 78 and represents a latent gammaherpesvirus infection with continuous but rare occurrences of viral antigen (Mistrikova et al., 2006).

In an initial effort we wanted to investigate whether exposure to hypoxia could affect production of MuHV-4 virions in the latently infected NB-78 cells. For the hypoxic treatment, the cells were plated on glass coverslips and incubated in anaerobic workstation (Ruskin Technologies, Bridgend, UK) in 2% O_2, 5% CO_2, 10% H_2 and 84% N_2 at 37 °C. Parallel samples were kept in the normal atmosphere (21% O_2) with 5% CO_2 at 37 °C. At each timepoint (0, 24, 48 and 72 h), the cells were fixed in ice-cold methanol for 10 min at −20 °C. All subsequent steps were performed at room temperature. Non-specific binding was blocked by incubation with PBS containing 0.2% Tween and 2% FCS for 60 min. The cells were then washed with PBS, incubated with rabbit polyclonal antibody produced against purified MuHV-4 and specific for five MuHV isolates (60, 68, 72, 76, 78, Svobodova et al., 1982). The antibody was diluted 1:100 in blocking buffer, cells were incubated with diluted antibody for 1 h, washed three times with PBS/0.2% Tween 20 and incubated with Alexa Fluor® 488 goat anti-rabbit IgG (Molecular Probes, Eugene, Oregon, USA) diluted 1:2000 in blocking buffer for 1 h. Following the final wash, the coverslips were mounted onto slides in mounting medium with Citifluor (Agar Scientific Ltd., Essex, UK) and analyzed by Leica DM 4500B epifluorescence microscope equipped with Leica DFC 480 digital camera. MHV-68-infected Vero cells grown under normal oxygen levels were used as a positive control. As reported earlier, the presence of viral antigens could be detected in about 1–2% of NB-78 cells under standard culture conditions (Mistrikova et al., 2006). Our immunofluorescence figure from normoxic NB-78 cells roughly corresponded to this report. However under hypoxic conditions, the number of cells expressing viral antigens was visibly higher at 48 h and further increased with the prolonged exposure (Fig. 1A). In addition, the number of MuHV-positive cells increased after 48 h exposure to hypoxia and additional 24–48 h cultivation under normal oxygen levels (data not shown).

To get more evidential data, we performed a similar experiment, where the number of MuHV-positive cells (expressing viral antigens on the cell surface) was determined by FACS-analysis, which is more suitable for quantitative assessments than immunofluorescence microscopy. NB-78 cells were grown on 60 mm plates, incubated under normoxic or hypoxic conditions for 0, 24, 48 and 72 h, trypsinized and stained for FACS analysis. Following trypsinization, cells were washed 3 times with FACS buffer (PBS with 2% fetal bovine serum and 0.05 NaN_3), incubated 30 min on ice with primary rabbit polyclonal antibody directed against MuHV-4 diluted 1:100, washed three times with FACS buffer, incubated 30 min on ice with secondary antibody Alexa Fluor® 488 goat anti-rabbit IgG (Molecular Probes, Eugene, Oregon, USA) diluted 1:1000 and washed again. Cells were fixed in 1% paraformaldehyde for 15 min at RT, washed, resuspended in PBS and the fluorescence was determined using a Coulter Epics Altra flow cytometer. By this staining about 7% of cells were considered positive for viral antigens at the 0 h timepoint. This number was not significantly changed after 24 h of hypoxic treatment, but 48 and 72 h exposure to hypoxia led to a significant increase in the number of cells expressing viral antigens (22% and 52%, respectively, Fig. 1B). Number of cells expressing viral antigens did not increase when cultivated in control (normoxic) conditions. Therefore, we concluded that MuHV-4 can reactivate from latently infected cells when exposed to hypoxic conditions. Our findings are in good agreement with KSHV, where hypoxia induces lytic virus replication in B-cell lines (Davis et al., 2001). Similar effect was recently described in hypoxia-treated B95-8 cells latently infected with EBV (Jiang et al., 2006). Thus, it seems that gammaherpesviruses evolved a strategy to escape from hostile micro-environment such as hypoxia.