Virology 401 (2010) 266–279

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Myxoma and vaccinia viruses exploit different mechanisms to enter and infect human cancer cells

Nancy Y. Villa [a], Eric Bartee [a], Mohamed R. Mohamed [a,c], Masmudur M. Rahman [a], John W. Barrett [b], Grant McFadden [a,*]

[a] Department of Molecular Genetics and Microbiology, College of Medicine, University of Florida, Gainesville, Florida 32610, USA
[b] Department of Microbiology and Immunology, BioTherapeutics Research Group, Robarts Research Institute, The University of Western Ontario, London, Ontario, Canada
[c] Department of Biochemistry, Faculty of Science, Ain Shams University, Cairo, Egypt

ARTICLE INFO

Article history:
Received 15 December 2009
Returned to author for revision
4 January 2010
Accepted 23 February 2010
Available online 24 March 2010

Keywords:
Myxoma
Vaccinia
Entry–fusion complex
Endocytosis
Macropinocytosis
PAK1
Genistein
Tyrosine kinase

ABSTRACT

Myxoma (MYXV) and vaccinia (VACV) viruses have recently emerged as potential oncolytic agents that can infect and kill different human cancer cells. Although both are structurally similar, it is unknown whether the pathway(s) used by these poxviruses to enter and cause oncolysis in cancer cells are mechanistically similar. Here, we compared the entry of MYXV and VACV-WR into various human cancer cells and observed significant differences: 1 — low-pH treatment accelerates fusion-mediated entry of VACV but not MYXV, 2 — the tyrosine kinase inhibitor genistein inhibits entry of VACV, but not MYXV, 3 — knockdown of PAK1 revealed that it is required for a late stage event downstream of MYXV entry into cancer cells, whereas PAK1 is required for VACV entry into the same target cells. These results suggest that VACV and MYXV exploit different mechanisms to enter into human cancer cells, thus providing some rationale for their divergent cancer cell tropisms.

Introduction

Poxviruses are large enveloped dsDNA viruses that replicate exclusively in the cytoplasm of infected cells (Moss, 2007). Historically, specific members of the different poxvirus genera have been more intensively studied than others because of their implications in human or veterinary diseases, or for use as vaccines, vectors or platforms for diverse therapies. For instance, variola virus (VARV), from the genus *Orthopoxvirus*, has been extensively studied as the causative agent of smallpox. VARV was eventually eradicated by vaccination with vaccinia virus (VACV), another related poxvirus of the genus *Orthopoxvirus* (Moss, 2007). VACV is one of the best studied and well characterized poxviruses, which has been used as a prototypical model to investigate poxvirus biology in general.

Recently, VACV has emerged as a potential oncolytic agent because of its rapid life cycle, strong target cell killing activity, inherent ability to preferentially replicate within tumor tissues (particularly for attenuated variants of VACV), large cloning capacity, well defined molecular biology, and its capacity to infect a variety of human cancer types (Kirn and Thorne, 2009; Thorne, 2008). A second poxvirus called Myxoma virus (MYXV), from the genus *Leporipoxvirus*, has also emerged as a potential oncolytic agent for treatment of human cancers (Stanford and McFadden, 2007). In contrast to VACV, which can productively infect a wide range of mammalian hosts, MYXV, can infect only lagomorphs. In European rabbits (*Oryctolagus cuniculus*), MYXV causes a lethal disease called myxomatosis (Fenner and Ratcliffe, 1965; Stanford et al., 2007). Although MYXV does not induce any known pathology in humans, or any other non-lagomorph host, this virus can efficiently replicate *in vitro* in a variety of transformed human cancer cells lines (Barrett et al., 2007a; Wang et al., 2006; Woo et al., 2008). The ability of MYXV to specifically infect and clear human cancer tissues *in vivo* has also been confirmed in immunodeficient mouse models using various xenografted human tumors (Lun et al., 2005, 2007; Wu et al., 2008). Because it has not been reported to induce any toxicity for humans, MYXV has also emerged as a promising candidate for virotherapy to treat a variety of human cancers, however unlike VACV, the molecular characteristics of MYXV infection have not been completely characterized.

One of the key tropism steps that can influence the ability of an oncolytic virus to distinguish normal cells from tumor cells is virus entry. Thus, elucidating and comparing the entry mechanism(s) used by MYXV and VACV to gain entry into human cancer cells is critical for advancing

* Corresponding author. Fax: +1 352 273 6849.
E-mail address: grantmcf@ufl.edu (G. McFadden).

0042-6822/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2010.02.027

these two poxviruses as oncolytic agents. Recently, a significant number of studies have shed light on the mechanism(s) used by VACV to bind and enter into host mammalian cells, the majority of which have been performed with the intracellular mature virion (MV) form of VACV, since this is the most abundant and stable infectious form (Moss, 2006). Initial binding of VACV MVs to mammalian cells occurs by mechanisms that can be dependent on either cell surface glycosaminoglycans (GAGs) or other still-unidentified cellular moieties (Carter et al., 2005; Chiu et al., 2007; Chung et al., 1998, 2005; Foo et al., 2009; Hsiao et al., 1999). Studies using electron microscopy originally demonstrated that the VACV MVs from the Western Reserve (WR) and modified vaccinia virus Ankara (MVA) strains enter cells by direct fusion with the plasma membrane (Carter et al., 2005). Later, it was confirmed that the fusion of VACV MV to the plasma membrane can occur at neutral pH, and is mediated by a multi-protein fusion complex carried by the virion (Moss, 2006). The number of viral proteins that comprise the poxviral entry–fusion complex (EFC) has continued to grow. For example, Satheshkumar and Moss (2009) provided recent evidence that VACV O3L (VACVWR069.5), a short open reading frame (ORF) of just 35 amino acids, which possesses orthologs in MYXV (Cameron et al., 1999) and molluscum contagiosum virus (Senkevich et al., 1997), is the newest identified integral component of the EFC, required for MV entry and membrane fusion.

Additionally, poxvirus MVs can also utilize a low-pH endosomal entry pathway (Townsley et al., 2006) but, interestingly, some strains of VACV cannot exploit this latter pathway (Bengali et al., 2009). A recent report suggests that VACV MV entry uses macropinocytosis, a transient growth factor-induced, and actin-dependent endocytic mechanism, which is utilized by large particles such as bacteria, apoptotic bodies, necrotic cells and viruses to penetrate into the cytoplasm of mammalian cells (Mercer and Helenius, 2008). The induction of macropinocytosis is preceded by the rapid activation of signaling pathways via tyrosine kinase receptors that ultimately trigger re-arrangements of actin filaments at the plasma membrane, which favors the internalization of interacting particles (Mercer and Helenius, 2009).

In contrast to VACV, the mechanism(s) whereby MYXV enters mammalian cells have yet to be studied in detail, although the known virus-encoded members of the viral EFC identified in VACV are highly conserved in MYXV. With this in mind, we first sought to establish if there were any differences in the entry mechanisms between the MYXV Lausanne (MYXV-Lau) strain and the VACV Western Reserve (VACV-WR) strain, using several human cancer cells as a model system. We report that substantial differences exist between these viruses with regard to the effects of low pH, and the requirement for endosomal acidification for entry. We used specific kinase inhibitors to demonstrate differences in the drug sensitivity to MV entry of both viruses. Specifically, we found that genistein, a tyrosine kinase inhibitor that blocks macropinocytosis, specifically inhibits the entry of MV particles from VACV, but not MYXV, into the same target cancer cells. These findings will further help our understanding of the various oncolytic mechanisms exploited by poxviruses and facilitate their development for oncolytic virotherapy.

Results

Replication of MYXV and VACV is cell-type dependent

Both MYXV and attenuated variants of VACV are potential oncolytic virus candidates for treatment of human cancers. In order to understand the mechanism of entry used by both viruses in various human cancer cells, we started this study comparing the infectivity of MYXV and VACV in several selected human cancer cell lines: A549 (lung carcinoma cells), HeLa (cervical carcinoma), Panc1 (pancreatic carcinoma) and BJAB (EBV-negative Burkitt B-cell lymphoma). We first used fluorescence microscopy to compare the ability of MYXV and VACV tagged with both EGFP (early/late expression) and Tomato Red (late expression only) to infect and spread within different cancer cell types. To do this, the indicated cells were infected with the recombinant vMyx-GFP-TrFP or VACV-GFP-TrFP at a MOI of 0.1 and evaluated by fluorescence microscopy 48 h post-infection (hpi). In each case, expression of GFP was a measure of early/late viral gene expression and TrFP was a measure of late gene expression only. We observed significant differences in infection progression by the viruses in some of the cancer cell types (Fig. 1). For instance, in A549 and HeLa cells, both MYXV (Fig. 1A) and VACV (Fig. 1B) appeared to initiate a permissive infection and underwent normal cell-to-cell spread. As well, the expected increase in the infectious progeny virus was observed for both viruses from A549 cells (Fig. 2A), which was comparable to titers in the control BGMK cells (Fig. 2B). In contrast, fluorescence microscopy analysis revealed the expression of both EGFP and TrFP from vMyx-GFP-TrFP and VACV-GFP-TrFP infection of BJAB (Figs. 1A and B, respectively), however, single step growth curves showed that infection of BJAB cells with MYXV did not produce new infectious virions while infection of these cells with VACV produced significantly fewer new virions than BGMK cells (Fig. 2C). The most notable differences between MYXV and VACV were observed with Panc1 cells, which supported productive replication for VACV but were completely nonpermissive for MYXV (Figs. 1A, B and 2D). Taken together, these results demonstrate that these viruses can differ significantly in terms of gene expression and progeny virus production in certain specific human cancer cells.

MYXV entry into cancer cells, unlike VACV, is not stimulated by acidic pH

The entry mechanism(s) used by the mature virions (MVs) of VACV have been actively investigated in recent years. In this regard, Vanderplasschen et al. (1998) first reported that, while VACV MVs can enter cells by fusing with the plasma membrane at neutral pH, VACV EEVs use a low pH as the entry pathway. Further studies conducted by Townsley et al. (2006) also demonstrated that VACV MVs can use the endocytic route via low-pH to enter to the cells,. This latter conclusion is based on the evidence that a brief low-pH treatment (e.g. pH 5.0) accelerates VACV MV entry into host cells. In contrast, the role of pH has not been previously tested for MYXV. To determine whether MYXV entry also exploits this low-pH endosomal route, the rate of virus entry was quantified at neutral and acidic pH by measuring luciferase expression from either vMyx-GLuc (expressing Gaussia luciferase from a synthetic early/late promoter), or VACV-FLuc (expressing firefly luciferase from the same promoter). This assay allowed us to compare the entry kinetics of each virus under different conditions.

In our initial studies, virus adsorption was synchronized by incubation of either, VACV-FLuc, or vMyx-GLuc to HeLa cells at 4 °C for 1 h. Then each virus was incubated at 37 °C to allow the synthesis of the indicated luciferases. Although under these conditions, we were able to readily measure the VACV-FLuc luciferase activity (Supplemental Fig. S1C), we did not detect any vMyx-GLuc luciferase activity (Supplemental Fig. S1A). Next, we tested the ability of both viruses to enter the cells at room temperature (25 °C), and observed similar results. Only when the adsorption of MYXV proceeded at 37 °C we were able to measure any viral luciferase activity (Supplemental Fig. S1A) indicating successful MYXV entry. To determine if this result was a peculiarity of HeLa cells, we next tested RK13 cells, a rabbit cell line permissive for MYXV and VACV. Virus entry was synchronized as described above. Surprisingly, we obtained the same results with RK13 cells (Supplemental Fig. S1, panels B and D) as with HeLa cells, suggesting that in contrast to VACV, MYXV has uniquely different temperature requirements for either binding and/or entry.

In order to evaluate the effect of low-pH on MYXV entry, purified vMyx-GLuc MVs were adsorbed to the cells at 37 °C and then were