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Double-faceted mechanism of parvoviral oncosuppression

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The H-1 parvovirus (H-1PV) exerts oncosuppressive action that has two components: oncotoxicty and immunostimulation. While many human tumor cells, including conventional drug-resistant ones, can be killed by H-1PV, some fail to support progeny virus production, necessary for infection propagation in neoplastic tissues. This limitation can be overcome through forced selection of H-1PV variants capable of enhanced multiplication and spreading in human tumor cells. In the context of further developing H-1PV for use in cancer therapy, arming it with immunostimulatory CpG motifs under conditions preserving replication and oncolysis enhances its action as an anticancer vaccine adjuvant. A first clinical study of H-1PV treatment in glioma patients has yielded evidence of intratumoral synthesis of the viral oncotoxic protein NS1 and immune cell infiltration.

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Introduction

Oncolytic parvoviruses belong to the species rodent protoparvovirus 1 and consist of an icosahedral protein capsid about 25 nm in diameter, containing a linear single-stranded DNA genome of approximately 5000 nucleotides. This genome comprises two transcription units, encoding nonstructural and capsid proteins, whose integrity is important for viral infectiousness [1]. The present review focuses on the oncolytic parvovirus H-1PV, whose natural host is the rat but which can replicate in and kill a number of tumor-derived human cells, while sparing their normal counterparts. As H-1PV also exerts tumor-suppressive action in various animal models (see [2,3] for recent reviews), it is being assessed and developed as a potential tool for cancer therapy and/or prevention. Its antitumor action has two components: oncotoxicity and anticancer immunostimulation. Its adjuvant effect depends, at least in part, on the immunogenicity of viral oncolysates, and is instrumental in mediating virus-induced anticancer vaccination in animal models (reviewed in [4–6]). This review aims to illustrate the duality of H-1PV antitumor action and to discuss prospects for its optimization through virus adaptation, engineering, and/or combination with other agents.

Parvovirus oncolytic effects and propagation

Oncotoxic activity of rodent protoparvoviruses

Oncolytic parvoviruses induce molecular disturbances that jeopardize the survival of infected tumor cells. Besides exhausting cell metabolites as a result of their multiplication, they encode proteins that may participate in shutting down cell DNA replication and gene expression, by binding and withholding cellular factors controlling these processes [2]. Some parvoviral products, such as the 83-kDa multi-function nonstructural protein NS1, appear to alter target cell integrity by directly or indirectly damaging the cytoskeleton, cytoplasmic organelles, and the plasma membrane [7–9,10••]. NS1 can notably bind to cellular transcription and replication factors and colocalize with nuclear DNA replication and repair components [11–16]. In addition, its presence in permissive tumor-derived or transformed cells leads to depolymerization of cytoskeletal micro-filaments and intermediate filaments [7,17], release of lysosomal cathepsins [9], accumulation of reactive oxygen species in the cytosol [18], and plasma membrane permeabilization [10••], resulting in apoptotic or non-apoptotic cell death [9,19,20].

Given the pivotal role of NS1 in oncolysis, its production and functioning are main determinants of parvoviral oncoselectivity, on which the use of parvoviruses in cancer therapy rests. There is evidence that intracellular changes contributing to malignant transformation stimulate NS1 synthesis [21], and cell transformation with oncogenes greatly enhances the intrinsic oncotoxicity of NS1 [22]. This modulation of NS1 activity is attributed to post-translational modifications — notably phosphorylation — of the NS1 protein [17,23]. Protein kinase Cs (PKCs), driving phosphorylation of specific NS1 residues,

are essential to NS1 functioning throughout infection and the eventual production of progeny virions [24–28]. In keeping with its role in the functional control of PKCs, the master kinase PDK1 appears as an important positive modulator of cell permissiveness toward parvovirus infection [7,29,30••]. Activation of PDK1/PKC/PKB signaling is a hallmark of malignant transformation, with effects on cell growth, differentiation, and survival [31,32]. We recently found PDK1 to be activated in a major proportion of tested glioma samples, through phosphorylation at a particular site, making the enzyme independent of cofactor PIP3 [30••]. We hypothesized that this could result in PKC-driven activating phosphorylation of NS1, leading to NS1-triggered virus replication and cell killing. Accordingly, we found expression of a constitutively active mutant form of PDK1 to be sufficient to sensitize H-1PV-resistant normal diploid human cells to virus-induced killing. The PDK1/PKC/PKB pathway is thus a determinant of parvovirus oncoselectivity, most likely acting through functional activation of NS1.

Although NS1 alone is sufficient to trigger cancer cell death in the absence of other viral products, natural productive infection is likely to display a different efficiency, kinetics, and pattern of cell killing. Interesting is our recent finding that, although lacking an envelope, progeny parvoviral particles are released through an active egress process involving their vesicular transport to the plasma membrane *via* the endoplasmic reticulum and Golgi apparatus [10••,33]. This mechanism controls the onset of cell lysis and favors rapid virus spread. Besides its impact on cell death, this exocytotic process may contribute to anticancer immunostimulation by bringing viral and tumor-associated intracellular proteins to the cell surface and promoting their presentation to the host immune system (see below).

Parvovirus adaptation to human cancer cells

Although oncolytic parvoviruses can kill various human tumor cells, infection is not always productive, that is, some infected cancer cells die without releasing progeny virions capable of spreading to neighboring uninfected tumor cells. This lack of virus propagation has notably been observed in a significant fraction of H-1PV-sensitive human glioma cell cultures [9,34] and may limit the overall efficacy of viral oncolysis and putative virus-egress-associated immunostimulation [35•].

We endeavored to isolate propagation-competent H-1PV variants through forced serial passages in a human-glioma-derived cell line (Figure 1a). We selected a pool of viral mutants (hgH-1PV) showing an enhanced capacity for productive infection of established (Figure 1b) and non-established (data not shown) human glioma cells. In a long-term cell killing assay, these hgH-1PV viruses destroyed human glioma cell cultures at lower multiplicities of infection than the original virus (Figure 1c). These 'humanized' hgH-1PV viruses also showed decreased infectivity toward glioma cells from rats (the natural host of H-1PV) (Figure 1b and c).

As illustrated in Fig. 1d (left and middle panels), the lower infectivity of the original H-1PV isolate toward human *versus* rat glioma cells correlates with underrepresentation of two specific NS1 phosphopeptides in the phosphopeptide profile of the former cells. In contrast, both phosphopeptides are prominent in human glioma cells infected with an adapted hgH-1PV virus (right panel). This, and the prior identification of both phosphopeptides as determinants of the capacity of NS1 to drive viral DNA amplification [28], led us to hypothesize that hgH-1PV viruses might harbor one or several mutations allowing NS1 to become functionally activated through phosphorylation in human glioma cells.

Immune mediation of H-1 parvovirus oncosuppression

There is growing evidence that the immune reactions triggered by non-pathogenic H-1PV depend largely on whether tumor tissues are present or not. Several *in vitro* studies have consistently shown that infection of human peripheral blood mononuclear cells (PBMCs) by H-1PV is not productive, although the first steps of viral replication do occur. More detailed analysis has identified B-cells, macrophages, and natural killer (NK) cells as the main immune cell subpopulations infected by H-1PV. In activated cells, infection causes induction of tumor necrosis factor (TNF)-alpha, interferons (IFN), or interleukins (IL) 2, 4, and 10 [36,37•]. A virus-related moderate deficit in immune cell proliferation is mainly caused by antiviral cytokines but not by viral cytotoxicity. This innocuousness of parvovirus infection toward the immune system was recently confirmed *in vivo* in a large number of rats ($n = 227$) having received multiple intravenous injections of high doses of highly purified H-1PV produced for clinical use in patients (up to 17 injections of 1×10^8 plaque-forming units over 28 days) [38•]. The animals developed neutralizing antibodies after H-1PV infection, showing no evidence of impaired B-cell activity. Serum cytokine levels (IL-6 and TNF-alpha) remained within normal limits, and the proliferative response of *ex vivo*-stimulated PBMCs from infected animals was similar to that of controls. Recent results likewise show no induction of an IFN response in human fibroblasts, glia, or melanocytes by parvovirus infection [39].

On the other hand, investigations of various components of the immune system have demonstrated that the antineoplastic action of H-1PV, initially due to intratumoral replication and oncolysis, depends strongly on successful immune stimulation. A first observation was that H-1PV-induced splenomegaly (a sign of lymphocyte proliferation) appeared only in tumor-bearing rats, but not in the absence of neoplastic tissue even after repeated injections