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Programmable insect cell carriers for systemic delivery of integrated cancer biotherapy

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ABSTRACT

Due to cancer's genetic complexity, significant advances in the treatment of metastatic disease will require sophisticated, multi-pronged therapeutic approaches. Here we demonstrate the utility of a *Drosophila melanogaster* cell platform for the production and in vivo delivery of multi-gene biotherapeutic systems. We show that cultured *Drosophila* S2 cell carriers can stably propagate oncolytic viral therapeutics that are highly cytotoxic for mammalian cancer cells without adverse effects on insect cell viability or gene expression. *Drosophila* cell carriers administered systemically to immunocompetent animals trafficked to tumors to deliver multiple biotherapeutics with little apparent off-target tissue homing or toxicity, resulting in a therapeutic effect. Cells of this Dipteran invertebrate provide a genetically tractable platform supporting the integration of complex, multi-gene biotherapies while avoiding many of the barriers to systemic administration of mammalian cell carriers. These transporters have immense therapeutic potential as they can be modified to express large banks of biotherapeutics with complementary activities that enhance anti-tumor activity.

Introduction

Genetically encoded biotherapeutics with naturally evolved activities can be harnessed for the treatment of disease. In particular, proteins with the ability to regulate cell growth, modulate the host immune system, or recognize tumor-specific antigens are of interest in the treatment of cancer. Although many tested agents demonstrate highly specific therapeutic effects when inoculated or expressed directly in tumors, they often lose potency when administered systemically due to poor stability in blood and/or rapid clearance from the circulation [1]. Incorporation of biotherapeutic genes into viral vectors can help increase the efficiency of delivery to the tumor site. In particular, conditionally replicating oncolytic viruses can be delivered systemically to tumors where they rapidly self-amplify to manufacture the therapeutic transgene specifically at the site of disease [2]. However, host immunity remains a major challenge to the clinical realization of this goal [3–5]. Intravenous delivery exposes oncolytic viruses to circulating factors such as neutralizing antibodies, which bind to and neutralize virus directly or mark them for destruction by complement and by various immune cells [6]. Virus is also neutralized by non-specific binding to serum proteins and circulating cells present in the bloodstream [7]. Organs such as the lungs, spleen, and especially the liver, also play a significant role in clearing virus as these tissues contain resident macrophages whose role is to scavenge the blood for circulating pathogens [7].

We have previously demonstrated that cellular carriers can shield oncolytic virus from neutralization to achieve systemic delivery to tumors in the presence of circulating antibodies [3]. This strategy has successfully been used to improve the delivery of various oncolytic virus platforms including adenovirus [8–10], measles virus [11–13], vesicular stomatitis virus (VSV) [3,14,15], reovirus [16–19], Newcastle disease virus [20], herpes simplex virus [21,22], parvovirus [23], retroviruses [24,25], myxoma virus [26] and vaccinia virus [27,28]. Clinical application and validation of this approach are currently ongoing [19,29].

Our group and others have investigated the ability of numerous mammalian cell types to function as oncolytic virus carriers for systemic therapy in pre-clinical models, including solid [3,23,28] and hematogenous [3,11] cell lines, cytokine-induced killer cells [27,30], T cells [13,21,25], primary monocytes [12,31], myeloid derived suppressor cells [15] and mesenchymal stem cells [32,33]. However, there remain significant obstacles to using any mammalian cell type for systemic oncolytic

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virus delivery in the clinical setting. Adherent solid tumor cells and mesenchymal stem cells are unable to traverse capillary beds and generally arrest within the vessels of the first organ they encounter [3,34], while leukocyte-based carriers are able to re-circulate but still exhibit receptor-mediated homing to lymphoid organs and bone marrow [3,35,36]. Thus interactions between mammalian carrier cells and off-target host tissues interfere with systemic tumor targeting. Secondly, primary cell types are often cumbersome to isolate and culture, while systemic administration of permanent cell lines carries the risk of tumorigenicity. Finally, oncolytic virus infection is by design cytotoxic to mammalian host cells. This precludes the opportunity to genetically modify virus-laden carriers and complicates clinical delivery.

In order to move beyond the limitations of mammalian cells, we have investigated the potential of Dipteran insect cells as novel vehicles for systemic biotherapeutic delivery. Given that VSV establishes a persistent, non-cytolytic infection in insect cells [37,38], we thought that this might simplify delivery and also enable further manipulation of the cell carriers to improve delivery and therapeutic activity. We chose to investigate *Drosophila* S2 cells as cell carriers since they are easy to culture, they grow to cell densities above 10^7 cells/mL, there exist several systems to engineer these cells to express foreign genes, and it has been shown that they support persistent infection of VSV [37]. Notably, we demonstrate that established *Drosophila melanogaster* cell lines supported continuous propagation of multi-gene oncolytic virotherapeutics normally cytotoxic to mammalian cells, enabling the simultaneous production of secondary biotherapeutic gene products. Systemically administered insect cell carriers were well tolerated, shielded virus from immune recognition and neutralization, circulated at stable levels with little off-target tissue homing, and effectively delivered oncolytic virus to tumors in immunocompetent animals. Systemic delivery of insect cell carriers was able to reduce tumor burden in a lung tumor model as well as a subcutaneous tumor model in immune competent mice. Established *D. melanogaster* cell lines therefore offer a genetically tractable platform suitable for systemic delivery of multiple integrated biotherapies.

Materials and methods

Cells

Human (HT29-colorectal adenocarcinoma, U2OS-osteosarcoma, 786-O-renal cell adenocarcinoma, HeLa cervical adenocarcinoma, SF268-astrocytoma, A549-lung carcinoma, MCF-7-mammary adenocarcinoma), murine (CT26-WT-colon carcinoma, CT26-lacZ-colon carcinoma, 4T1-mammary carcinoma, L1210-lymphocytic leukemia, A20-B-cell lymphoma), BHK-21 (baby hamster kidney fibroblast) and Vero (African green monkey kidney epithelial cells) cell lines were cultured in Dulbecco's modified eagle medium (DMEM, Hyclone) supplemented with 10% fetal calf serum (FCS) (Hyclone). All mammalian cells were cultured at 37 °C under 5% CO_2. *D. melanogaster* Schneider line 2 (S2) cells were cultured in SF900II serum-free medium (Invitrogen) at 25 °C under atmospheric pressures. Establishment of murine L1210 leukemia cells expressing an integrated firefly luciferase transgene (L1210-FLuc) has been described previously [3]. All cell lines were obtained from the American Type Culture Collection (ATCC). S2-VSVΔ51YFP, S2-VSVΔ51-mRFP, and S2-VSVΔ51-FLuc represent *Drosophila* S2 cells persistently infected with the following viruses: VSVΔ51-YFP, VSVΔ51-mRFP, VSVΔ51-FLuc, respectively. S2-VVdd-mCherry represents S2 cells infected with vaccinia virus VVdd-mCherry and S2-VSVΔ51-YFP/VVdd-mCherry represents persistently infected S2-VSVΔ51YFP cells superinfected with VVdd-mCherry. S2-SINV-GFP represents S2 cells persistently infected with SINV-GFP and S2-SINV-GFP/VVdd-mCherry represents S2-SINV-GFP cells superinfected with VVdd-mCherry.

Viruses

Construction of recombinant strains of VSVΔ51 expressing firefly luciferase or monomeric red fluorescent protein reporter transgenes (referred to herein as VSVΔ51-FLuc and VSVΔ51-mRFP, respectively) have been described previously [3,39]. An additional recombinant harboring a yellow fluorescent protein (YFP) reporter was generating by subcloning the YFP coding region between the XhoI and NheI sites of the pXN vector [40] and rescuing recombinant virus as described previously [39]. All VSV stocks were propagated on Vero cells. For animal studies, VSV stocks were further purified from cell culture supernatants by filtration through a 0.22 μm Steritop filter (Millipore) and centrifugation at 30,000 $\times g$ before resuspension in phosphate-buffered saline (PBS) (Hyclone). Vaccinia (VVdd-mCherry) was made by insertion of mCherry-DNA into the vaccinia thymidine kinase (TK) gene locus of VVdd [41] by homologous recombination. Successful recombinants were selected by mCherry expression and plaque-purified. Vaccinia stocks were propagated in U2OS cells and cell-associated virus was collected by repeat (3) freeze–thaw cycles. Further purification of viral stocks was done by centrifugation at 20,700 $\times g$ through a 36% sucrose cushion (in 1 mM Tris) before resuspension in 1 mM Tris, pH 9. Sindbis virus expressing GFP, referred to as SINV-GFP, was propagated in BHK-21 cells.

Infection and preparation of insect cell carriers

Drosophila S2 cells were initially infected with VSVΔ51 at a multiplicity of 10PFU/cell (PFU: plaque forming units). Cultures were passaged continually at a density between 10^6–10^7 cells/mL to maintain log-phase growth. Doubly -infected carriers were generated by superinfecting S2-VSVΔ51 carriers with VVdd-mCherry at a multiplicity of 10 for 24 h. For delivery to tumor cells, insect cells were washed three times in 10 mL SF900II medium, and resuspended in a final volume of PBS before inoculation onto cell monolayers or injection into animals.

Cell viability assay

CT26-LacZ tumor cells were infected with VSV purified either from mammalian Vero cells or from *Drosophila* S2 cells and cell viability was assessed by alamarBlue (Life Technologies) assay according to the manufacturer's protocol.

Western blot

Cell lysates were collected in 4% sodium dodecyl sulfate sample buffer, run on a NuPAGE Bis-Tris 4–12% polyacrylamide gel, transferred to a PVDF membrane, and probed with polyclonal anti-VSV serum from hyperimmune rabbits. An anti-tubulin (clone YL1/2) antibody (Novus Biologicals) was used as a loading control.

Analysis of cultured insect cells

Cell suspensions were counted on an automated cell viability analyzer (Beckman Coulter), which determined the total cell concentration, viability by trypan blue exclusion, and average cell diameter.

Preparation of insect cell conditioned medium

Drosophila S2 cells were either mock infected or infected with VVdd-mCherry at a multiplicity of 10 PFU/cell for 24 h, harvested and then pelleted by centrifugation. Supernatants were collected and passed through a 0.22 μm filter twice to eliminate cell-free vaccinia virions. To test for factors enhancing VSV infectivity, tumor cell monolayers were pre-treated for 2 h with conditioned insect cell supernatant diluted into an equal volume of DMEM + 10% FCS. Tumor cells were then infected with VSV in the presence of conditioned medium.