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Delivery of oncolytic adenovirus into the nucleus of tumorigenic cells by tumor microparticles for virotherapy

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ABSTRACT

Oncolytic viruses have been utilized for the treatment of various cancers. However, delivery of the viral particles to tumor cells remains a major challenge. Microparticles (MP) are vesicle forms of plasma membrane fragments of 0.1–1 μm in size that are shed by cells. We have previously shown the delivery of chemotherapeutic drugs using tumor cell-derived MPs (T-MP). Here we report that T-MPs can be utilized as a unique carrier system to deliver oncolytic adenoviruses to human tumors, leading to highly efficient cytolysis of tumor cells needed for *in vivo* treatment efficacy. This T-MP-mediated oncolytic virotherapy approach holds multiple advantages, including: 1) delivery of oncolytic adenovirus by T-MPs is able to avoid the antiviral effect of host antibodies; 2) delivery of oncolytic adenovirus by T-MPs is not limited by virus-specific receptor that mediates the entry of virus into tumor cells; 3) T-MPs are apt at delivering oncolytic adenoviruses to the nucleus of tumor cells as well as to stem-like tumor-repopulating cells for the desired purpose of killing them. These findings highlight a novel oncolytic adenovirus delivery system with highly promising clinical applications.

1. Introduction

Oncolytic virotherapy has shown great potential to suppress tumor growth at different levels. Viruses are manipulated to selectively replicate and assemble in tumor cells, leading to the lysis of infected tumor cells. As a result, the released progeny viruses further infect neighboring tumor cells and amplify the lytic efficiency [1,2]. Moreover, tumor-invading oncolytic viruses combined with the lysed tumor cells attract a large number of immune cells, causing an acute inflammatory reaction at the tumor site. Typically, dendritic cells migrate to such inflammatory site to capture, process and present tumor antigens to T cells, leading to tumor-specific T cell immunity. Such antitumor immunity even overrides the virus-mediated lysis, conferring a durable therapeutic outcome [3–6]. Currently, oncolytic HSV (herpes simplex virus) has been approved by the FDA for the treatment of advanced melanoma [7,8]. Other oncolytic viruses such as vaccinia, adenovirus, reovirus and VSV (vesicular stomatitis virus) have also clinically developed to phase I or phase II stage [1–3]. Yet, notwithstanding the significant progress in this field, oncolytic virotherapy is still facing a number of obstacles [9–12], including rapid clearance of oncolytic viruses by pre-existing or induced antiviral antibodies; downregulation of virus-recognizing receptors that limits the virus entering tumor cells; activation of intracellular antiviral defense mechanisms

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against entered viruses; and inefficacy in controlling multifocal advanced diseases or metastasized tumors. In addition, oncolytic virotherapy may cause certain side effects. For instance, oncolytic VSVs are beset by risks of neuropathy, including paralysis or lethal encephalitis [13]. Therefore, an improved oncolytic virus delivery system that overcomes those impediments is highly desirable. By their tumor tropism ability, mesenchymal stem cells, myeloid-derived suppressor cells, cytokine-induced killer cells or even irradiated tumor cells have been used to deliver oncolytic viruses [13–16]. In addition, synthesized nanoparticles are also used to coat viruses as carriers [17]. However, all these strategies have their own limitations. For example, when they arrive at the tumor site, carrier cells may need to be lysed for releasing of inside viruses; and the synthesized nanoparticles which contain 'nonself' components elicit immune rejection [18].

Recent studies highlight that cells are capable of generating various vesicles with different sizes. In response to stimuli, cells change their cytoskeleton, leading to encapsulation of cytosolic contents within cellular membrane to form vesicles and their subsequent release into extracellular spaces. These specialized subcellular vesicles with 0.1–1 μm sizes are called microparticles (MPs) [19,20]. MPs not only contain messenger molecules, enzymes, RNAs and even DNA, but also are capable of transferring these bioactive molecules from one cell to another [21–24]. We have recently demonstrated that tumor cell-derived MPs (T-MP) can serve as safe and efficient carriers to deliver chemotherapeutic drugs to tumor cells [25]. Moreover, T-MPs show their inherent anti-tumor nature by containing both tumor antigens and innate signals [26].

Adenoviruses are the most widely used oncolytic agents in clinical settings with high safety profiles. Oncolytic adenoviruses can selectively infect tumor cells regardless of cell cycling and induce an S phase-like state that facilitates viral replication [27–29]. However, adenoviruses are extremely immunogenic and prone to be cleared by immune mechanisms [30]. In this study, we hypothesize that T-MPs can function as a natural viral delivery platform to evade immune attack and facilitate adeno-oncolytic efficacy and efficiency. We provide evidence that T-MPs are unique carriers with multiple advantages in delivering oncolytic adenoviruses, implying potential clinical applications.

2. Materials and Methods

2.1. Mice and cell lines

C57BL/6, BALB/c and nude mice, 6 to 8 week-old, were purchased from Centre for Experimental Animal Research for studies approved by the Institutional Animal Care and Use Committee of Peking Union Medical College. All the cell lines were purchased from Cell Resource Centre of Peking Union Medical College (Beijing, China), and cultured according to the given guidelines.

2.2. Construction and preparation of adenoviruses

AdMaxTM recombinant adenovirus system (Microbix Canada), which consists of a shuttle plasmid pDC515 and adenoviral backbone pBGHfrtΔE1,3 FLP, was used for the construction of adenoviruses. To construct a replication-defective adenovirus expressing enhanced green fluorescent protein (EGFP), the EGFP fragment was amplified from pEGFP-C1 (ClonTech) by PCR and inserted into pDC515. The construct was named as pDC515 EGFP.

To construct oncolytic adenovirus type 5, a recombinant DNA fragment that contains human telomerase promoter and adenoviral E1A cDNA fragment (hTERTp-E1A) was amplified from pDC-hTERTp-E1A-IRES-TK plasmid [31]. hTERTp-E1A was then used to replace the CMV promoter of pDC515 to yield pDC-hTERTp-E1A.

For packaging Ad5 EGFP and Ad5 hTERTp-E1A, the shuttle plasmids pDC515 EGFP and pDC-hTERTp-E1A were co-transfected with backbone pBGHfrtΔE1,3 FLP into HEK 293 cells, respectively, according to the instruction of AdMaxTM recombinant adenovirus system. Crude viruses from the HEK 293 cells were purified and quantified by adenovirus purification and quantitation kits, respectively (Cell BioLabs Inc).

2.3. Preparation of OA-MPs

1×10^7 A549 cells were infected with 1×10^8 VP of OA. 48 h later, when the most cells detached from the culture dishes, the culture media were collected for OA-MP isolation as described before [25]. In brief, supernatants were first centrifuged for 10 min at 600 *g* to get rid of cells and then centrifuged for 2 min at 14,000 *g* to remove debris. At last, the supernatant was further centrifuged for 60 min at 14,000 *g* to pellet OA-MPs. To generate control MPs, uninfected tumor cells were exposed to ultraviolet irradiation (UBV, 300 Jm^{-2}) for 1 h and continued 12-h culture. The supernatants were used for control MP isolation.

2.4. MP counting

A flow cytometry-based method was used to count the number of MPs. After centrifugation, the MPs were suspended with PBS that was pre-filtered through 0.1 μm filter and passed through 1 μm filter to further exclude background noise or nonspecific events. The MPs mixed evenly with 3 μm latex beads (LB-30, Sigma, St Louis, MO) with a known number. For flow cytometric analysis, 0.8 μm deep-blue dyed-latex beads (L1398, Sigma) were first used for gating and voltage adjustment, as such beads are fluorescent and can be detected on FL4 channel. When the mixture was analysed by flow cytometry, each LB30 bead formed a dot in the gate of the large-size population. If 10,000 counts of LB30 were collected, the number of MP can be calculated with formula: $N = 10{,}000 \times (MP\%/LB30\%)$.

2.5. Nucleic acid extraction, PCR and RT-PCR

For DNA extraction, all the samples were first digested with protease K (20 mg/ml) at 37 °C overnight. The solution was extracted with phenol/chloroform and the top aqueous layer was transferred for DNA isolation. On the other hand, total RNA was extracted with TRIzol (Invitrogen) according to the manufacturer's instructions. RT-PCR was performed using the One-Step RT-PCR Kit (Toyobo). The oligonucleotides are as follows: *E1A*, sense 5-CCGCTCGAGAGACATATTATC TGCCACGG-3, antisense 5-GGGGTACCTGGCCTGGGGCGTTTACA-3; *fiber*, snese 5-ATGAAGCGCGCAAGACCGTCTG-3, antisense 5-CCTGGACCAGTTGC TACGGTC-3; *hexon*, sense 5-ATGGCTACCCCTTCGATGATGCC-3, antisense 5-A CCATTGCCTGCGGTCGCCT-3; human *β-actin*, sense 5-GCACCACACCTTCTAC AATGAG-3, antisense 5-GGTCTCAAACATGATCTGGGTC-3.

2.6. Adenoviral genomic DNA quantification

Adenoviral genomic DNA was isolated from OA-MPs with the High Pure Viral Nucleic Acid Kit (Roche). The qPCR primers for hexon DNA were designed (sense: 5′-ATGATGCCGCAGTGGTCTTA-3′; antisense: 5′-GTCAAAGTACGTGGAAGCC AT-3′). The qPCR was carried out in a 20 μl reaction volume under the following conditions: 1 μl of DNA sample, 1 μl of forward primer, 1 μl reverse primer, 10 μl of SYBR-Green Real-time PCR Master Mix and 7 μl of dH_2O. Quantification of adenoviral copy number was performed