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Proteasome inhibition in cancer is associated with enhanced tumor targeting by the adeno-associated virus/phage

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ABSTRACT

Bacteriophage (phage), which are viruses that infect bacteria only, have shown promise as vehicles for targeted cancer gene therapy, albeit with poor efficiency. Recently, we generated an improved version of phage vectors by incorporating cis genetic elements of adeno-associated virus (AAV). This novel AAV/phage hybrid (AAVP) efficiently delivered systemically administered therapeutic genes to various tumor targets by displaying an integrin tumor-targeting ligand on the phage capsid. However, inherent limitations in bacteriophage mean that these AAVP vectors still need to be improved. One of the limitations of AAVP in mammalian cells may be its susceptibility to proteasomal degradation. The proteasome is upregulated in cancer and it is known that it constitutes a barrier to gene delivery by certain eukaryotic viruses. We report here that inhibition of proteasome improved targeted reporter gene delivery by AAVP in cancer cells *in vitro* and in tumors *in vivo* after intravenous vector administration to tumor-bearing mice. We also show enhanced targeted tumor cell killing by AAVP upon proteasome inhibition. The AAVP particles persisted significantly in cancer cells *in vitro* and in tumors *in vivo* after systemic administration, and accumulated polyubiquitinated coat proteins. Our results suggest that the proteasome is indeed a barrier to tumor targeting by AAVP and indicate that a combination of proteasome-inhibiting drugs and AAVP should be considered for clinical anticancer therapy.

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1. Introduction

Cancer gene therapy has faced a problem common to all forms of gene therapy, namely the lack of a systemically-administered, safe and efficient vector to deliver the gene of interest at the diseased site. Animal viruses have been shown to be capable of ligand-targeted gene delivery, but they require the elimination of their native tropism for mammalian cells so that they can be re-targeted at alternative receptors (Hajitou et al., 2006a; Hajitou, 2010), which results in reduced efficacy (Allen et al., 2006; Ghosh and Barry, 2005; Hajitou et al., 2006a; Hajitou, 2010). Incorporation of targeting peptides

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derived from *in vivo* phage display screenings into viral vectors has been attempted, but with little success because either the ligand destroys the vector or the vector destroys the ligand (Ghosh and Barry, 2005; Hajitou, 2010). A solution to this limitation may be to use bacteriophage as the gene delivery vehicle (Hajitou et al., 2006a; Hajitou, 2010; Larocca et al., 1998; Poul and Marks, 1999). This eliminates the need to transfer peptides from phage display to a eukaryotic virus, and no native tropism for mammalian cells needs to be circumvented. Bacteriophage are safe and can be targeted by a ligand displayed on their capsid to a specific mammalian receptor after systemic administration (Hajitou, 2010). Unfortunately, phage particles are considered to be poor vectors, nevertheless, as they have evolved to infect bacteria only and, therefore, have no intrinsic strategies for delivering genes to mammalian cells.

To overcome this limitation, we have recently generated an improved version of such phage-based vectors as hybrids between two single stranded DNA viruses; adeno-associated virus (AAV) and M13 phage (termed AAV/Phage; AAVP). Within this novel vector, a targeted phage capsid serves as a vehicle to deliver a recombinant rAAV mammalian DNA cassette incorporated into an intergenomic region of the bacteriophage genome (Hajitou et al., 2006b). This vector showed superior gene delivery compared to a regular phage vector with long-term gene expression *in vivo* after systemic delivery (Hajitou et al., 2006b). We found that this improved mammalian transduction efficiency is associated with several factors: the improved fate of the delivered gene through maintenance of the entire mammalian transgene cassette, better persistence of episomal DNA, and formation of concatamers of the AAV transgene cassette (Hajitou et al., 2006b, 2007). In these previous studies, we used AAVP displaying the cyclic RGD4C (CDCRGDCFC) peptide ligand to target overexpressed α_v integrins in tumors. Therapeutic genes were successfully delivered to the tumor site in mice and rats while sparing the normal organs after intravenous administration (Hajitou et al., 2006b, 2007, 2008; Tandle et al., 2009; Trepel et al., 2009). A recent study carried out under the direction of the National Cancer Institute of the USA has elegantly confirmed the potential of this technology (Paoloni et al., 2009). Targeted AAVP was used to deliver a cytokine, tumor necrosis factor-α (TNFα), to cancers diagnosed in pet dogs. Repeated doses proved safe and resulted in complete eradication of aggressive tumors in some of these dogs (Paoloni et al., 2009).

It is clear that AAVP represent a new generation of phage-based vectors that have promise. However, due to inherent limitations of bacteriophage, they need to be improved to enable them to overcome intracellular barriers in mammalian cells. Phage internalization assays have shown that 100% of cells *in vitro* internalize the targeted phage via a receptor-mediated endocytosis, only as few as 10% of cells actually express the transgene (Hajitou et al., 2007). This is probably due to the fact that, unlike eukaryotic viruses, bacteriophage have no strategies to evade the barriers to infective agents that mammalian cells present. Proteasomes are one of these barriers. They are multi-subunit enzymes responsible for the degradation of many cytosolic proteins (e.g. misfolded proteins, cyclins, and transcription factors) and for processing foreign proteins prior to the deployment of cellular immune responses (Groll et al., 1997; Kisselev, 2008; Tanaka et al., 2012). Numerous previous studies have reported the proteasome as an obstacle to some eukaryotic viral vectors (Monahan et al., 2010). Furthermore, cancers possess an elevated level of proteasome activity (Chen and Dou, 2010; Kisselev, 2008; Wu et al., 2010). It would therefore seem likely that the activity of proteasomes represents one possible barrier to the efficient delivery of AAVP vectors to cancer cells.

We report here an investigation of the efficacy of targeted gene delivery by RGD4C/AAVP to cancer in the presence of the proteasome inhibitors, MG132 and the Calpain 1 inhibitor LLnL. The MG132 and LLnL are peptide aldehyde inhibitors that reversibly inhibit the 26S proteasome activity (Kisselev and Goldberg, 2001; Lu et al., 2006; Masdehors et al., 2000; Vinitsky et al., 1992), and most widely used in proteasome inhibition studies (Gartel, 2010; Granot et al., 2007; Lu et al., 2006). We found that combination of proteasome inhibitors with RGD4C/AAVP resulted in significantly improved reporter gene expression *in vitro* and *in vivo*, and better tumor cell killing than the vector alone. Next, we established that this improved efficacy is associated with better persistence of the AAVP particles both *in vitro* and *in vivo* and with increased polyubiquitination of the AAVP coat proteins, when used in combination with a proteasome inhibitor. Our results strongly suggest that supplementary proteasome inhibition should be considered as the potential of AAVP vectors is further explored.

2. Material and methods

2.1. *Reagents and cells*

The Human Embryonic Kidney (HEK293) cell line was purchased from American Type Culture Collection (ATCC). Human M21 Melanoma cells were a gift from Dr David Cheresh (University of California, La Jolla), the human U87 glioblastoma cells were from Cancer Research UK and rat 9L glioblastoma cells were provided by Dr Hrvoje Miletic (University of Bergen, Norway). All these cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) supplemented with 10% Fetal Bovine Serum (FBS, Sigma), Penicillin (100 units/ml, Sigma), Streptomycin (100 μg/ml, Sigma) and L-Glutamine (2 mM, Sigma). Cells were cultured in a humidified atmosphere of 37 °C in a 5% CO_2 and passaged every 3–4 days when they reached 80–90% confluence. To assess tumor cell killing *in vitro*, cells were incubated with medium containing ganciclovir (GCV) at 20 μM. GCV was renewed daily and cells were counted using the trypan blue-exclusion methodology.

2.2. *MTT assay*

Mitochondrial activity (a measure of cellular viability) was measured with the MTT (3,4,5-dimethylthiazol 2,5 diphenyltetrazolium bromide) assay and was used to determine the viability of cells following treatment with Z-Leu-Leu-Leu-al (MG132, Sigma) and the Calpain 1 inhibitor N-Acetyl-Leu-Leu-Norleu-al (LLnL, Sigma). Cells were plated at a density of 4×10^3 cells/well in a 96 well plate (Nun C). Stock solutions of 4 mM for MG132 and 40 mM for LLnL were prepared by using dimethyl sulfoxide (DMSO, Sigma). Cells were treated with

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