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Enhanced transduction of CAR-negative cells by protein IX-gene deleted adenovirus 5 vectors

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ABSTRACT

In human adenoviruses (HAdV), 240 copies of the 14.3-kDa minor capsid protein IX stabilize the capsid. Three N-terminal domains of protein IX form triskelions between hexon capsomers. The C-terminal domains of four protein IX monomers associate near the facet periphery. The precise biological role of protein IX remains enigmatic. Here we show that deletion of the protein IX gene from a HAdV-5 vector enhanced the reporter gene delivery 5 to 25-fold, specifically to Coxsackie and Adenovirus Receptor (CAR)-negative cell lines. Deletion of the protein IX gene also resulted in enhanced activation of peripheral blood mononuclear cells. The mechanism for the enhanced transduction is obscure. No differences in fiber loading, integrin-dependency of transduction, or factor-X binding could be established between protein IX-containing and protein IX-deficient particles. Our data suggest that protein IX can affect the cell tropism of HAdV-5, and may function to dampen the innate immune responses against HAdV particles.

Introduction

Protein IX is a non-essential protein in the capsid of human adenoviruses (HAdV). The protein has a size of 14.3 kDa, is present at 240 copies per virion, and has three highly conserved regions present in the amino (N) terminus, the central part (alanine-rich), and the carboxy (C) terminus (leucine-rich). The location and function of protein IX in the virus capsid has been the subject of investigation and debate for many years (Vellinga et al., 2005b). Recent work by different groups has brought consensus on its location and topology in the capsid (Fabry et al., 2009; Saban et al., 2006). The N-terminus of the protein is located in between hexon cavities of the groups of nine (GON) hexons, presumably stabilizing the GONs. The C-terminus of the protein forms an alpha helix and is exposed on the capsid surface in close contact with hexon hypervariable region 4 (HVR4) (Saban et al., 2006). C-terminal domains of three protein IX molecules associate in a parallel orientation, whereas a fourth domain binds in an antiparallel orientation (Fabry et al., 2009). The role of protein IX in the capsid remains enigmatic. *In vitro* analysis revealed the N-terminus of protein IX to confer a thermostable phenotype on HAdV-5 capsids (Vellinga et al., 2005a). Propagation of protein IX gene deleted HAdV-5 in cell culture yields wild-type like virus titers, demonstrating that protein IX is dispensable for virus replication *in vitro*.

Protein IX has potential as an anchor for the attachment of different types of polypeptides to the viral capsid. Targeting of HAdV-5 to tumor cells has been achieved by genetically fusing protein IX to a single-chain T cell receptor directed against MHC class I in complex with MAGE-A1 peptides (de Vrij et al., 2008). Similarly, integrin-binding arginine–glycine–aspartate (RGD) peptides, as well as single-chain antibody fragments have been incorporated in this way (Vellinga et al., 2007, 2004). Alternatively, targeting ligands can be coupled to protein IX via the genetic inclusion of cysteine residues and subsequent chemical coupling of ligands to the reactive thiol groups (Corjon et al., 2008). Multiple polypeptides can be incorporated simultaneously (Tang et al., 2009). A triple-mosaic HAdV-5 vector was developed with a poly-lysine motif, the herpes simplex virus type 1 (HSV-1) thymidine kinase, and the monomeric red fluorescent protein fused with protein IX, thereby combining targeting, therapeutic, and imaging modalities. Recently, it was demonstrated that HAdV-5 vaccine vectors with pathogen-specific antigens fused to pIX can stimulate robust protective immune responses in animals, suggesting a new route for the development of improved HAdV-5 based recombinant vaccines (Bayer et al., 2010; Boyer et al., 2010).

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Here we report on the enhanced delivery of transgenes into CAR-negative cell lines as a result of protein IX-gene deletion from a HAdV-5-based vector. Furthermore, the protein IX-deficient particles demonstrated enhanced activation of peripheral blood mononuclear cells (PBMCs), and had a different *in vivo* distribution after intravenous delivery in a mouse model. The exact molecular mechanism behind this 'ΔpIX effect' remains to be delineated. Our data suggest that protein IX can affect the cell tropism of HAdV-5, and may function to dampen the innate immune responses against HAdV particles.

Results

Enhanced transgene expression in CAR-negative cells with Ad5ΔE1ΔpIX

To study the role of protein IX in the HAdV-5 transduction of cells, we compared the vectors Ad5ΔE1+pIX and Ad5ΔE1ΔpIX for luciferase transgene expression in a panel of cell lines (Fig. 1A). Cell lines with varying expression levels of CAR were included (Fig. 1B). Whereas similar expression levels were obtained with both vectors in the CAR-positive cell lines HeLa, A549, and MEL2A, the vector Ad5ΔE1ΔpIX yielded higher levels than Ad5ΔE1+pIX in the CAR-negative cell lines MZ2-MEL3.0 and VH10. Since these results suggested a specific role of the protein IX lacking vector in mediating relatively higher transduction in the absence of CAR, Ad5ΔE1+pIX and Ad5ΔE1ΔpIX were analyzed for reporter gene expression in MZ2-MEL3.0 cells versus MZ2-MEL3.0/CAR cells (Fig. 2B). MZ2-MEL3.0/CAR cells stably expressed CAR via transduction with a recombinant lentivirus, which was confirmed by flow cytometry and immune-fluorescence staining (Fig. 2A). In MZ2-MEL3.0 cells the reporter gene expression upon infection with Ad5ΔE1ΔpIX was found to be ten-fold increased compared to infection with Ad5ΔE1+pIX, while in MZ2-MEL3.0/CAR cells the difference was a mere two-fold (Fig. 2B). The enhanced transgene expression for Ad5ΔE1ΔpIX on the CAR-negative cell line MZ2-MEL3.0 appeared to be not affected by the establishment of protein IX expression in the cells (by using the recombinant lentivirus LV-CMV-pIX-IRES-NPTII; Vellinga et al., 2006) prior to the transduction) (result not shown).

As a next step, the involvement of the C-terminal region of protein IX in the observed phenomenon was investigated. This domain, which is rich in leucine amino acids and is exposed on the HAdV-5 capsid as an alpha-helical structure (Fabry et al., 2009; Saban et al., 2006), is highly conserved in human adenoviruses. The biological function of this conserved domain of protein IX is unknown. We analyzed the vector Ad5ΔE1pIX$^{\Delta LEU}$, which lacks a major part of the C-terminal region of protein IX (amino acids 100 to 114) for reporter gene expression in MZ2-MEL3.0 and MZ2-MEL3.0/CAR. Ad5ΔE1pIX$^{\Delta LEU}$ demonstrated enhanced transduction of the CAR-negative cell line, very similar to the Ad5ΔE1ΔpIX vector (Fig. 2C).

To assess the appearance of the vector particles and to check for the absence of microaggregation, electron microscopy was performed on Ad5ΔE1+pIX and Ad5ΔE1ΔpIX vector batches. This showed identically shaped virus particles (Fig. 3A). No signs of microaggregation were observed. The Ad5ΔE1ΔpIX stock appeared to contain more small particulate matter, possibly virus debris. As previously described, pIX-deficient HAdV-5 particles have an enhanced tendency to partly dissociate into fiber- and penton base-lacking particles (Fabry et al., 2005). However, our vectors had similar capsid incorporation levels of fiber and hexon proteins, as evident from immunoblot analyses (Fig. 3B), thus ruling out differences in particle dissociation for the vector preparations.

Transduction with Ad5ΔE1ΔpIX is integrin-dependent

Wild-type HAdV-5 enters cells via high affinity binding of the fiber knob domain to CAR (Bergelson et al., 1997). Subsequently low affinity interaction of the penton base with cellular integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$ promotes virus internalization in clathrin-coated pits (Nemerow and Stewart, 1999; Wickham et al., 1993). To answer the question if Ad5ΔE1ΔpIX still uses integrins for cellular uptake, we analyzed Ad5ΔE1+pIX and Ad5ΔE1ΔpIX for transgene expression (GFP) in the presence or absence of bivalent cations, which are necessary for integrin-mediated uptake of wild-type HAdV-5 into cells (Wickham et al., 1993) (Fig. 4A). This experiment again displayed a stronger reporter gene expression of Ad5ΔE1ΔpIX in MZ2-MEL3.0 cells compared to Ad5ΔE1+pIX. For both vectors the transduction appeared to be totally dependent on the presence of bivalent cations, with a complete reduction to background GFP levels for the cation-negative incubation. This is consistent with integrin-mediated uptake for both vectors. More specifically, the integrin-dependency of Ad5ΔE1ΔpIX was confirmed by a small but significant (approximately two-fold)

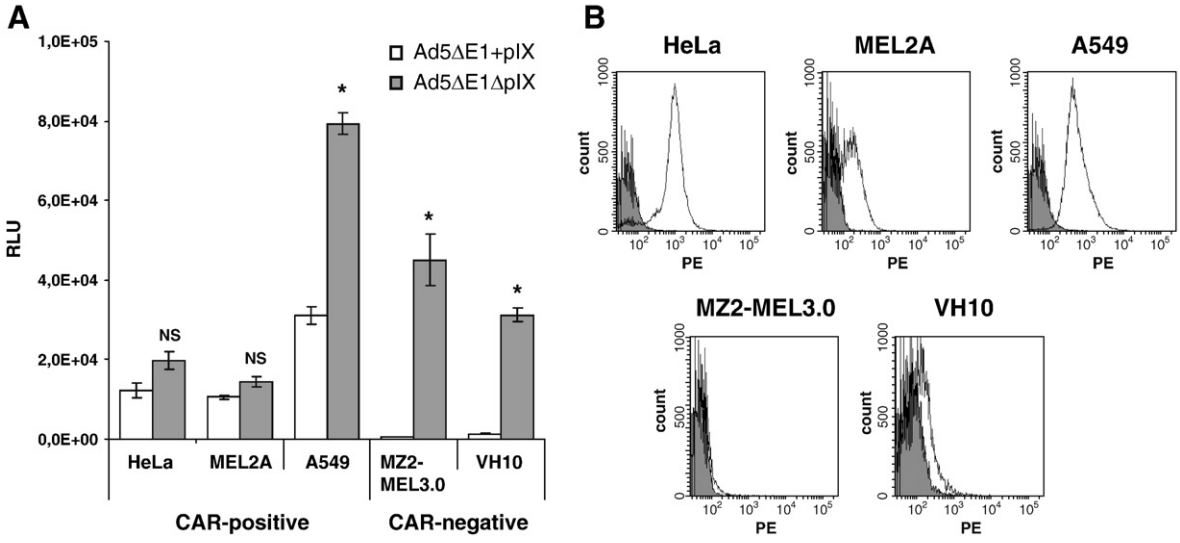

Fig. 1. (A) Transduction of CAR-positive and CAR-negative cells with the replication-deficient vectors Ad5ΔE1+pIX and Ad5ΔE1ΔpIX. At 24 h post transduction (at 10 pp/cell) the luciferase expression was measured as indicated by the relative luciferase units (RLU) (NS signifies Not Significant, *$p<0.02$ versus Ad5ΔE1+pIX). Error bars represent SEM ($n=3$). (B) Flow cytometry with anti-CAR antibody and PE-labeled secondary antibody to analyze cell surface expression level of CAR (white histograms). The gray histograms represent incubation with secondary antibody only.