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Helper-dependent adenovirus vectors devoid of all viral genes cause less myocardial inflammation compared with first-generation adenovirus vectors

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Abstract *Background* First-generation, E1-deleted (ΔE1) adenovirus vectors currently used in cardiovascular gene therapy trials are limited by tissue inflammation, mainly due to immune responses to viral gene products. Recently, helper-dependent (HD; also referred to as "gutless") adenovirus vectors devoid of all viral coding sequences have been shown to cause low inflammation when injected intravenously or into skeletal muscles. However, HD vectors have not been evaluated in cardiovascular tissues. *Methods and results* HD and ΔE1 vectors containing a cytomegalovirus-driven expression cassette for the green fluorescent protein (GFP) gene were administered intramyocardially to adult rats (n = 54). GFP expression was measured by ELISA at varying time intervals after gene transfer. HD and ΔE1 vectors were equally efficient at transducing the myocardium. Tissue inflammation was assessed by immunostaining for leukocytes and quantitative real-time RT-PCR for cytokine mRNA expression. Monocyte/macrophages, $CD4^+$ and $CD8^+$ lymphocytes infiltrating the myocardium were less abundant with HD than ΔE1 vectors. Transcripts levels for pro-inflammatory cytokines such as IL-1β, tumor necrosis factor-α, and RANTES were decreased with HD vectors. However, both vectors were associated with a decline in GFP expression over time, although low-level expression was occasionally detectable 10 weeks after HD vector administration. The two vectors transduced endothelial cells in rat arteries (n = 11) with comparable efficiencies. Vascular GFP expression was not detectable at 10 weeks. *Conclusions* HD vectors are as efficient as ΔE1 vectors at transducing the myocardium and vascular endothelium, while causing less myocardial inflammation. Thus, HD vectors may be superior to earlier-generation adenovirus vectors for cardiovascular gene therapy applications.

Key words High-capacity – helper-dependent – gutless – adenovirus – heart

Introduction

Efficiency and safety of gene transfer vectors are key determinants of the clinical success of gene therapy. Recombinant adenovirus vectors have been used in a number of cardiovascular gene therapy studies and clinical trials because they efficiently transduce both cardiomyocytes and vascular cells *in vivo* [13, 36]. Moreover, adenovirus has been used safely as a life vaccine in large populations, with no evidence of tumor induction. First-generation adenovirus vectors deleted in the E1 early gene region (ΔE1), with or without deletion of the dispensable E3 early region (ΔE3), are currently used in

gene therapy trials for coronary artery disease [16, 32]. However, usefulness of ΔE1 and ΔE1/ΔE3 vectors is similarly limited by tissue inflammation and short-lived transgene expression. The E3 region contains open reading frames (ORFs) for viral proteins that have anti-apoptotic (e.g., E3-10.4K, E3-14.5K, E3-14.7K) and immunomodulatory activities (e.g., E3-gp19K). Second-generation adenovirus vectors carrying constitutive expression cassettes for E3-14.7K or E3-gp19K have been constructed. The E3-14.7K vector injected into the liver mediated cytoprotective effects in mice, but did not affect persistence of adenovirus DNA [15]. Similarly, we showed reduced arterial inflammation after gene transfer with an E3-gp19K vector in rabbits, but duration of transgene expression was not significantly prolonged [40]. ΔE1 and ΔE1/ΔE3 vectors still contain E2 and E4 early adenoviral genes as well as late genes coding for immunogenic proteins that induce cytotoxic immune reactions [41, 42]. Consistent with a major role of immune responses for short-lived transgene expression with these vectors, we showed that treatment with immunosuppressive drugs prolongs transgene expression in rat arteries [36].

Severe inflammatory reactions to adenovirus vectors have been observed experimentally in the lung [5], liver [42], brain [24], and heart [13]. Determinants of vector toxicity include the vector backbone, the transgene product itself, and the purity of vector preparations. Clinical-grade purity is superior to that of vectors used in most experimental studies. Adverse events directly related to adenovirus vectors have been relatively rare in clinical gene therapy trials [19]. However, a young patient died in 1999 soon after receiving adenovirus-based gene therapy for treatment of partial ornithine transcarbamylase (OTC) deficiency [20]. After receiving the highest dose of the vector in the trial, the patient developed acute respiratory distress syndrome and died two days later of multiple organ failure. The death was ascribed to an unusually strong inflammatory reaction to the vector.

As an experimental tool to assess the effect of ΔE1/ΔE3 vectors in the presence of negligible production of viral proteins, we previously used UV-irradiation, which suppresses viral genes expression without substantially affecting the viral capsid. UV-irradiated adenovirus vectors caused less inflammation and achieved longer gene expression compared with non-irradiated vectors in rat arteries *in vivo* [36]. More recently, adenovirus vectors devoid of most or all viral coding sequences (so-called "gutless" vectors) have been developed to minimize viral gene products [6, 12, 18, 22, 23, 30, 33]. Gutless vectors contain only the elements required *in cis* for virus replication and packaging; hence, they need co-infection with a helper virus that provides all necessary viral proteins *in trans* for growth *in vitro*. Such helper-dependent (HD) vectors injected intravenously provided for efficient and sustained transgene expression with decreased inflammation in the liver in mice [8, 10, 27, 29, 34], dogs [8], and baboons [28]. Also, they efficiently transduced skeletal muscles in mice [6, 25]. While the helper virus load of HD vector preparations was of major concern with earlier production techniques [1, 12, 26], improved systems based on a strong biological selection against the helper virus have been developed more recently [18, 30]. The biological selection is based on the excision of the packaging signal, which is flanked by loxP sites, from the helper virus genome by Cre-recombinase expressed by the producing cells during co-infection.

Low vector toxicity and sustained transgene expression are desirable features in most gene therapy strategies. We therefore compared HD with first-generation adenovirus vectors in cardiovascular gene transfer models. The two vectors were equally efficient at transducing the myocardium and vascular endothelium; however, myocardial inflammation was markedly attenuated with HD vectors.

Methods

HD vector system

The HD adenovirus vector system was provided by Merck. Plasmid pC4HSUgfp contains the HD adenovirus backbone, a cytomegalovirus (CMV)-driven expression cassette for the green fluorescent protein (GFP) reporter gene, and stuffer DNA comprising fragments of human genomic DNA selected by screening for the absence of genes, repeat elements, and coding sequences, as described [33]. Helper virus H14 contains a modified packaging signal φ flanked by two loxP sites, and 2,902 bp of human DNA, as described [34].

HD vector rescue and purification

HD vector production was carried out in 293cre4 cells expressing Cre-recombinase (Merck) [7]. Cells were transfected in six-well plates with 3 μg of *Pme*I-digested pC4HSUgfp using the FuGene reagent (Roche). Twelve hours after transfection, 293cre4 cells were infected with H14 helper virus at a multiplicity of infection (MOI) ≈ 5. Wells that reached cytopathic effects at 48 h were selected for propagation into passage 2. Cells were lysed by three freeze/thaw cycles, and 2 ml of lysate were used to infect a 10-cm dish of 293cre4 cells together with helper virus. Infection cycles were repeated with increasing numbers of cells until passage 6. At passage 6, HD vector from fifty 15-cm plates was purified by triple CsCl banding.

HD vector titration and purity

The number of GFP gene-transducing particles in HD vector preparations was determined as followed: 293 cells were infected with serial dilutions of the HD vector preparation. For comparison, 293 cells were infected at different defined MOIs with the ΔE1 CMV-GFP vector to generate a standard curve. Cells were incubated for 15 hours, and percentages of GFP-expressing cells were determined by flow cytometry. Helper virus was titered by using an end-point dilution method in 96-well plates. Plates were seeded with 3×10^3 293 cells that were infected 24 hours later with serially diluted virus (range: 10^{-7}–10^{-10}). Infected cells were incubated for 14 days, and cells with cytopathic effects were scored visually.

First-generation adenovirus vector

The ΔE1/ΔE3 CMV-GFP adenovirus vector (referred to as ΔE1 vector) was constructed, propagated in 293 cells, and purified by triple CsCl banding, as described [36].

Myocardial gene transfer

Animal procedures were approved and carried out according to institutional guidelines. Male Sprague-Dawley rats (12–16 week-old; IFFA-Credo, L'Arbresle, France) were anesthetized with i.p. ketamine/xylazine (66.7/6.7 mg/kg), intubated, and mechanically ventilated. A thoracotomy was performed in the 5th left intercostal space, and vector containing solution (6×10^7 transducing units; TU, diluted in PBS to a final volume of 25 μl) was injected with a 32-gauge needle into the cardiac apex and the apical portion of the anterior LV wall. Myocardial GFP expression was determined by ELISA at varying time intervals after gene transfer with HD (n=18) or ΔE1 vectors (n=12). Injection of virus dilution buffer alone served as a control (n=3). Additional rats (n=6) receiving low-dose (≈8×10^6 TU) HD vectors were sacrificed at 1 and 2 weeks (n=3 each). In an attempt to reactivate the CMV promoter within HD vectors, additional rats (n=6) were re-thoracotomized 10 weeks after gene transfer. Retinoic acid (RA; 100 nM) and trichostatin A (TSA; 32 nM) [14], or PBS (n=3 each), were injected into the cardiac apex, and hearts were harvested 4 days later for GFP-ELISA. Myocardial inflammation was assessed on additional hearts receiving either HD vector, ΔE1 vector (2×10^7 TU each, diluted in virus dilution buffer to a final volume of 25 μl; n=3 and 4, respectively), or virus dilution buffer alone (n=3 each). Vectors were injected into the apex and the apical portion of the anterior LV wall under visual control (change in myocardial color). Hearts were harvested 12 days later for RT-PCR for cytokine expression analysis and for immunohistochemistry of inflammatory cell infiltrates.

GFP-ELISA

Hearts were excised, weighed, and homogenized. Total cytoplasmic proteins were extracted using the NE-PER™ kit (Pierce). GFP concentrations in cardiac extracts were measured by ELISA using React-bind™ anti-GFP coated plates (Pierce). Briefly, 100-μl samples in Assay Diluent for OptEIA™ (PharMingen) were coated for 1 hour at RT. Plates were washed and rabbit anti-GFP serum (IgG fraction; Molecular Probes) was added (60 min), followed by goat anti-rabbit biotinylated Ab (60 min), streptavidin-HRP (45 min), and TBM substrate. The reaction was stopped with 100 μl H_2SO_4 1.8 N. Plate reading was performed at 450 nm. A standard curve (0.9–4,000 pg/ml) with recombinant GFP (Molecular Probes) was made. Background values in hearts injected with virus dilution buffer were subtracted from those in transduced hearts.

Immunohistological analysis

Immunohistochemistry was performed on cryosections from hearts harvested 12 days after gene transfer. Briefly, rats were sacrificed by lethal pentobarbital injection, perfused with PBS, and the heart were excised. The cardiac apex was kept on ice-cold PBS for RT-PCR, while a thick myocardial slice directly adjacent to it was cut parallel to the cardiac short axis and immediately frozen in OCT-compound. This slice contained the distal part of the needle track and the vector injection site, as visualized on histological sections. In previous experiments with ΔE1 adenovirus vectors using the same injection technique, we had observed a wide distribution of GFP expressing myocytes across this injected area, even at distant sites from the needle track. The tissue sampling protocol used in the present study was based on those previous observations. Inflammatory cells infiltrating the myocardium were analyzed in three series of 8-μm sections per heart, cut at fixed 100-μm steps starting from the apex. Immunostainings were performed with the following mAbs: anti-ED1-like (1C7) for monocyte/macrophages, anti-TCRαβ (R73), anti-CD8α (OX-8; all from PharMingen), anti-CD4 (W3/25; Accurate Chemicals), irrelevant mouse mAb MOPC-31C (PharMingen), and biotin-conjugated rabbit $F(ab')_2$ anti-mouse Ig (Jackson Immuno Research Laboratories), followed by StreptABComplex/HRP and DAB/H_2O_2 (Dako). Morphometric analysis was performed in 6–8 microscopic fields (10X) per cardiac section using the NIH-Image-1.62 program.

Quantitative real-time reverse transcriptase-PCR

Hearts were excised and about 200 mg of apical myocardium was placed in ice-cold PBS and cut into thin slices, then submerged into RNAlater Stabilization

Reagent. Total RNA was extracted with the Qiagen RNeasy midi kit. DNase-treated RNA was used to generate cDNA, using the reverse transcriptase Omniscript (Qiagen), random hexamer (Promega) and RNase inhibitor (Roche). cDNA equivalent to 100 ng of total RNA was used for each PCR reaction. Transcript levels for cytokines were measured by quantitative real-time PCR (Rotor-Gene 2000, Corbett Research), using the SYBR-green dye. Constitutively expressed hypoxanthine phosphoribosyltransferase (HPRT) was selected as endogenous control to correct for potential variation in RNA loading or efficiency of the amplification reaction. Primer sequences were as follows: interferon-γ (IFN-γ), 5'-tcatggccctctctggctgttac-3' (sense) and 5'-caagaggaggctctttccttccatag-3'(antisense); interleukin-1β (IL-1β), 5'-cttcaaatctcacagcagcatctcg-3' (sense) and 5'-tccacgggcaagacataggtagc-3' (antisense); tumor necrosis factor-α (TNF-α), 5'-ctgtgcctcagcctcttctcattc-3'(sense) and 5'-ttgggaacttctcctccttgttgg-3'(antisense); transforming growth factor-β (TGF-β), 5'-ctaatggtggaccgcaacaacg-3'(sense) and 5'-tctggcactgcttcccgaatg-3' (antisense); RANTES, 5'-ccatatggctgcgacaccactc-3' (sense) and 5'-gcacacacttggcggttcctt-3' (antisense); HPRT, 5'-ctcatggactgattatggacaggactg-3' (sense) and 5'-cagcgctttaatgtaatccagcaggtc-3' (antisense). The threshold cycle (C_T) was defined as the fractional cycle number at which the reported fluorescence reached a defined level [3]. Target gene normalized to HPRT was expressed as ΔC_T (C_T of target gene minus C_T of HPRT). For amplicons optimized according to the manufacturer's guidelines, amplification efficiency is typically close to one, i. e., product accumulation increases twofold until the plateau phase is reached. Thus, the ratio of target gene/HPRT could be calculated by $2^{-\Delta CT}$. ΔC_T values obtained in the HD vector group were further normalized to the ΔE1 vector group by subtracting the ΔC_T values obtained in this group from those obtained with HD vectors, yielding $\Delta\Delta C_T$ [2].

Arterial gene transfer

Arterial gene transfer was performed in male Sprague-Dawley rats anesthetized with ketamine/xylazine. The left carotid artery was surgically exposed, and a silicone catheter was introduced through an arteriotomy of the external carotid artery. Virus vector stocks were diluted to a final concentration of 7×10^8 TU/ml with M-199 medium supplemented with 1 mg/ml rat albumin fraction V (Sigma). Virus-containing medium was instilled into the isolated carotid segment, where it remained for 20 minutes. Blood flow was then reestablished through the common and internal carotid arteries.

Histological analysis of arteries

Rat carotid arteries were harvested 5 days (n = 4 for each vector) or 10 weeks after gene transfer (n = 3 for HD vector), rinsed in PBS, and snap-frozen in OCT compound. Ten arterial sections per vessel, cut at a fixed distance of 100 μm from each other, were examined for GFP expressing cells by direct fluorescence microscopy. Corresponding sections were stained with hematoxylin/eosin.

Statistical analysis

Data are shown as median values (range), unless stated differently. Differences in peak GFP levels (1 week after gene transfer) and in cytokine mRNA expression between HD and ΔE1 vectors were compared with the Wilcoxon test (rank sums). Differences in myocardial inflammatory cell infiltrates among the three experimental groups (HD vectors, ΔE1 vectors, and buffer solution alone) were compared with the Kruskal-Wallis test (rank sums). The Wilcoxon test was used for 2 group-analysis of immunohistochemical data. p-values < 0.05 were considered to be statistically significant.

Results

HD vector preparation

Transducing titers for HD and ΔE1 vector stock preparations were 2×10^9 and 3×10^{10} TU/ml, the number of virus particles were 3.5×10^{10} and 3×10^{11} particles/ml, with calculated virus particles/TU ratios of 17.5 and 10.0, respectively. Purity of HD vector preparations was 99.8 %.

HD vectors efficiently transduce rat myocardium

HD vectors efficiently transduced rat myocardium *in vivo*. Cardiac myocytes accounted for the majority of GFP expressing cells, as identified based on their characteristic striated morphology (Fig. 1). At varying time intervals after high-dose (7×10^7 TU) vector injection, GFP concentrations in cardiac extracts were measured by ELISA and expressed as median values (range) normalized per gram of tissue. One week after gene transfer, cardiac GFP concentrations were comparable with HD and ΔE1 vectors: 59.2 ng (45.8 – 67.1 ng) vs. 55.3 ng (41.2 – 58.5 ng; NS). However, GFP levels fell ≈6-fold to 9.2 ng (1.8 – 18.2 ng) 2 weeks after gene transfer with HD vectors (Fig. 2B). Using low-dose (8×10^6 TU) HD vectors, GFP levels were 0.9 ng (0.2 – 2.8 ng) and 1.2 ng (0 – 32.8 ng) at

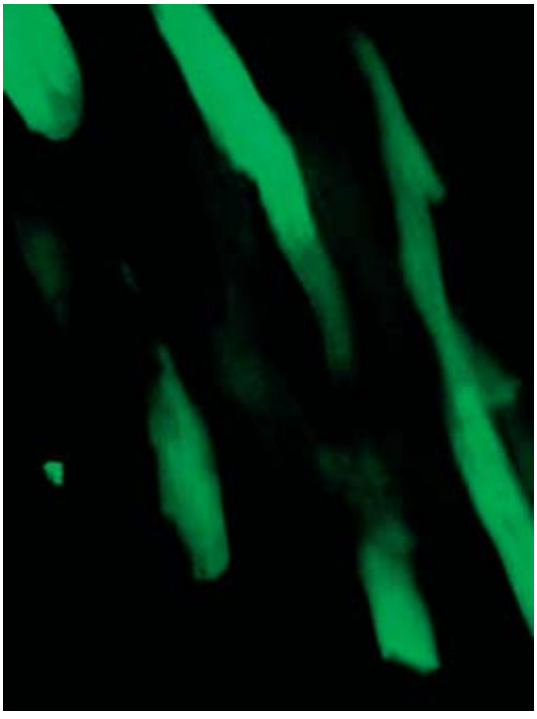

Fig. 1 Photomicrograph showing GFP expression in rat cardiomyocytes after HD vector-mediated *in vivo* gene transfer. The GFP expressing cells were readily identified as myocytes based on the characteristic morphology (original magnification, 500x)

1 and 2 weeks, respectively. Using ΔE1 vectors, cardiac GFP levels were 50.7 ng (1.0 – 73.1 ng) and 0.9 ng (0.8 – 1.1 ng) at 2 and 4 weeks, respectively (Fig. 2A). Ten weeks after gene transfer, GFP was barely detectable (threshold ≈0.05 ng) in most hearts, with the exception of one heart (1.6 ng) in the HD vector group. These results indicate that HD vectors efficiently transduce adult myocardium in a dose-dependent manner. However, transgene expression is short-lived in most cases. In the subgroup of mice re-thoracotomized 10 weeks after gene transfer with HD vectors, significant GFP levels (0.3 and 2.8 ng) were detected in 2 out of 3 hearts injected with RA+TSA, but in none of those injected with PBS. Modest pharmacological reactivation of the CMV promoter driving transgene expression may occur in some hearts late after gene transfer; however, our data are not conclusive in this regard.

HD vectors are associated with attenuated cellular inflammation

To evaluate myocardial inflammation, we immunostained sections from hearts injected with HD vectors, ΔE1 vectors or buffer solution alone for multiple inflammatory cell markers (Fig. 3A). A small area of disturbed tissue structure, corresponding to a small fibrotic lesion induced by intramyocardial needle injection, along with GFP-positive cells (Fig. 1) documented appropriate tissue sampling. Significant differences among the 3 experimental groups were found for ED-1–like$^+$, CD8α$^+$, and TCRαβ$^+$ cells ($p<0.05$ for each marker using the Kruskal-Wallis test). HD vectors caused markedly less monocyte/macrophage and T-cell infiltrates than ΔE1 vectors, as evidenced by ≈5-fold decreased ED-1–like$^+$ myocardial cell population (6.3 ± 3.9% vs. 32.4 ± 5.1%), ≈7-fold decreased CD4$^+$ population (5.9 ± 3.5% vs. 43.1 ± 4.2%), ≈5-fold decreased TCRαβ$^+$ population (5.7 ± 3.4% vs. 30.1 ± 4.4%), and ≈5-fold decreased CD8α$^+$ population (5.5 ± 3.4% vs. 29.4 ± 4.8%) ($p<0.05$ for each marker using the Wilcoxon test; Fig. 3B). TCRαβ$^+$ cells, but not ED-1–like$^+$, CD8α$^+$ and CD4$^+$ cells, were more abundant with HD vectors compared with

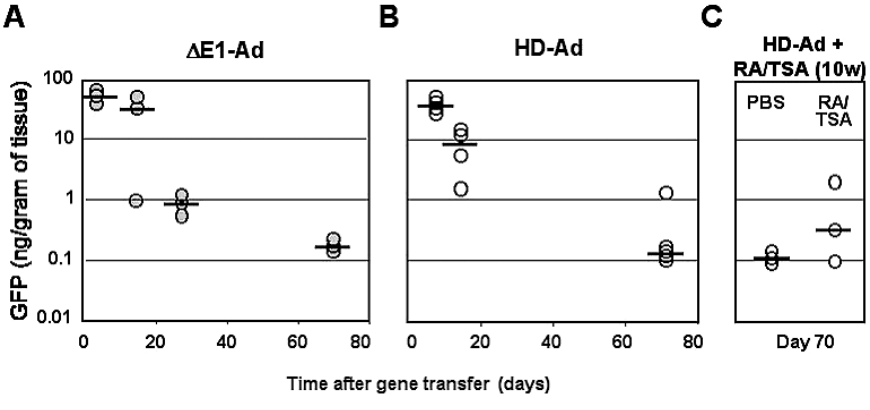

Fig. 2 **A** GFP content in cardiac extracts at varying time intervals after gene transfer with ΔE1 vectors (determined by ELISA). **B** GFP content in hearts injected with HD vectors. **C** GFP content in hearts injected with RA/TSA or PBS 10 weeks after HD vector injection, 4 days before analysis. Individual (circles) and median values (horizontal lines) are shown for each time point. Peak GFP levels with the two vectors were observed 1 week after gene transfer and were comparable to each other; however, they rapidly declined thereafter. Intramyocardial RA/TSA administration at 10 weeks was associated with detectable GFP expression in 2 out of 3 hearts injected with HD vectors. Data are nanograms GFP normalized for 1 gram of tissue (logarithmic scale)

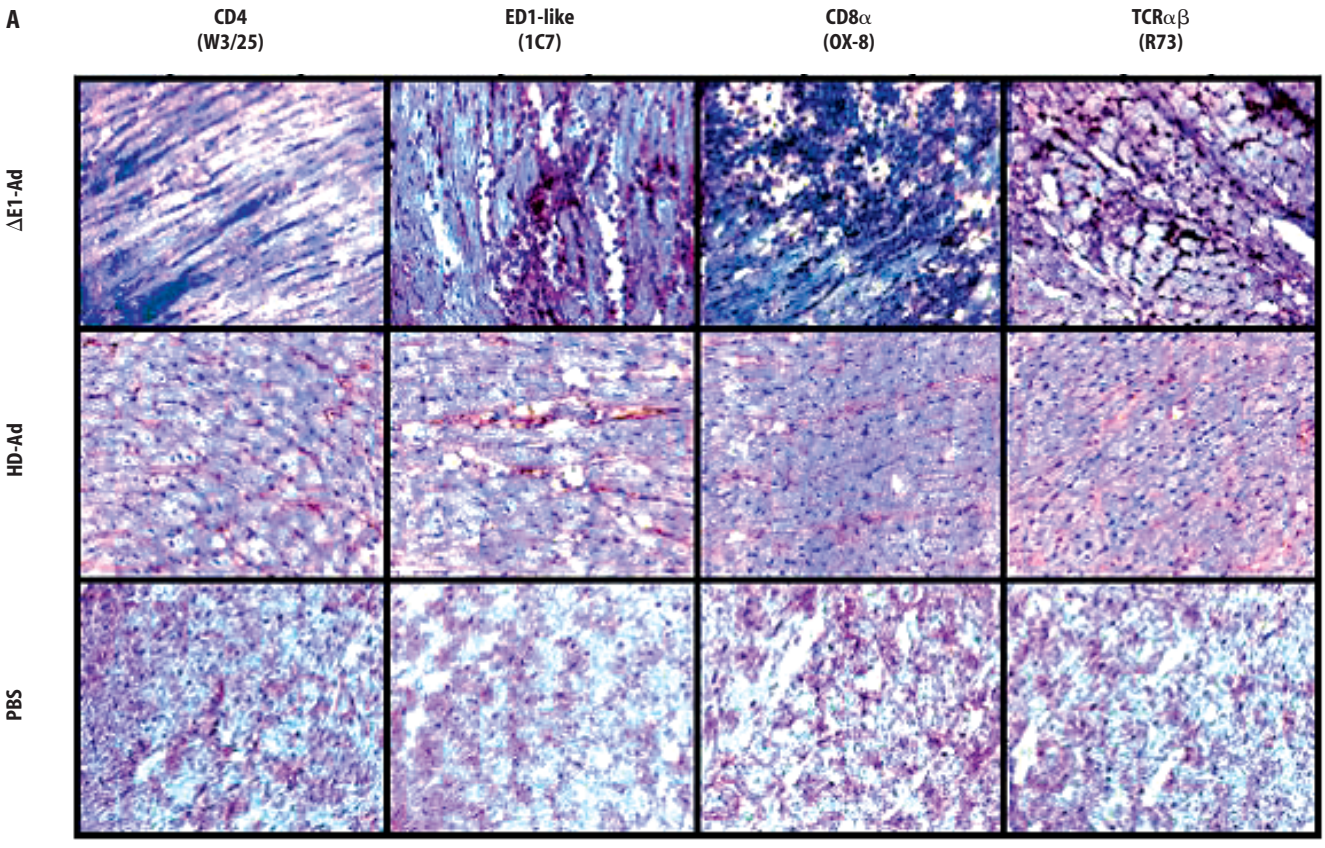

Fig. 3 **A** Inflammatory cells infiltrating the myocardium after gene transfer with ΔE1 (upper row) and HD vectors (middle row). Control hearts received virus dilution buffer (PBS; lower row). Immunostainings for CD4, ED1-like, CD8α, and TCRαβ (columns from the left to the right; original magnification, 100x). **B** Quantitative analysis of inflammatory cell markers. Macrophages and T cells were less abundant with HD compared with ΔE1 vectors. Data are mean percent positive-staining myocardial areas ±SD. § = $p < 0.05$ with the Wallis-Kruskal test (3 group-analysis); * = $p < 0.05$ with the Wilcoxon test (rank sums; 2 group-analysis)

buffer solution alone. These results indicate that HD vectors cause markedly less cellular inflammation than ΔE1 vectors, although they induce some residual, low-level inflammation.

HD vectors are associated with attenuated expression of pro-inflammatory cytokines

mRNA expression of several cytokines was determined by real-time RT-PCR in hearts injected with HD or ΔE1 vectors (Fig. 4). HD vectors induced significantly decreased transcript levels (expressed as $2^{-\Delta\Delta CT}$, normalized to ΔE1 vectors) for the pro-inflammatory cytokines IL-1β (0.01; range, 0 – 0.01; $p < 0.05$), TNF-α (0.01; range, 0 – 0.02; $p < 0.05$), and the RANTES chemokine (0.04; range, 0.03 – 0.05; $p < 0.05$). Transcripts of IFN-γ (median value) were reduced by ~90% with HD compared with ΔE1 vectors, but differences were below the significance level. Transcripts of TGF-β were slightly (not significantly) increased with HD compared with ΔE1 vectors. Enhanced expression of TGF-β as an immunomodulatory molecule may inhibit tissue inflammation.

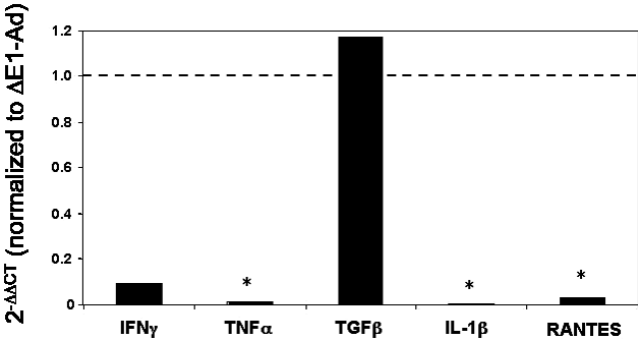

Fig. 4 Cytokine mRNA expression in cardiac extracts with HD vectors normalized for cytokine expression with ΔE1 vectors. Data are $2^{-\Delta\Delta CT}$ (values above and below the dotted line = 1.0 show induced and inhibited cytokine expression, respectively, using HD vectors relative to that with ΔE1 vectors (logarithmic scale; * = $p < 0.05$)

HD vectors transduce vascular endothelium *in vivo*

HD vectors were tested in rat carotid arteries *in vivo*. Both HD and ΔE1 vectors selectively transduced endothelial cells in uninjured vessels (Fig. 5; transduction rates ≈3–5% with either vector). A dose-dependent increase in transduction rates (up to ≈30%) was observed with ΔE1 vectors (data not shown). However, this aspect could not be assessed with HD vectors due to the lower titer of these vectors' preparations, which reflects current limitations of vector production techniques. GFP expressing endothelial cells were not detectable 10 weeks after gene transfer.

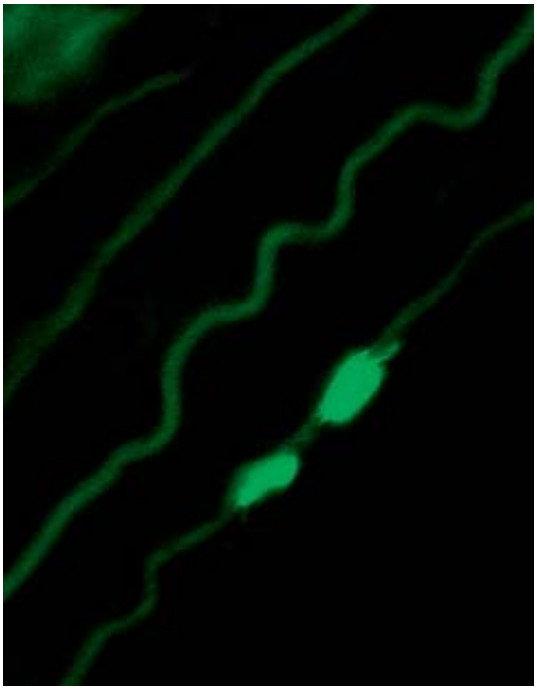

Fig. 5 HD vector-mediated GFP gene transfer into a rat artery. Two GFP-expressing endothelial cells are visualized by direct fluorescence microscopy (original magnification, 500x)

Discussion

First-generation adenovirus vectors have been used in many cardiovascular studies and in phase I/II gene therapy trials for ischemic heart disease and peripheral vascular disease. A large body of experimental evidence, along with the rare occurrence of adverse events in clinical trials suggest that these vectors are associated with significant toxicity. Immune responses to viral proteins produced by first-generation ΔE1 and ΔE1/ΔE3 adenovirus vectors cause tissue inflammation with consequent clearance of transduced cells. Recently, improved adenovirus vectors devoid of all viral coding sequences ("gutless" vectors) have been developed.

In the present study, we show that HD and ΔE1 vectors are equally efficient, on a virus particle-basis, at transducing adult myocardium and arterial endothelium *in vivo*. Myocardial gene transfer was dose-dependent. HD vectors caused markedly less myocardial inflammation than ΔE1 vectors, as reflected by lower numbers of monocyte/macrophages, total T cells, $CD4^+$ and $CD8^+$ lymphocytic infiltrates, as well as decreased mRNA expression for pro-inflammatory cytokines such as IL-1β, TNF-α, and RANTES. These results are in good agreement with previous data showing modest to negligible HD vector toxicity in the liver and skeletal muscle [6, 8, 10, 25, 27–29, 34]. However, myocardial GFP expression was short-lived in the present study, even though low-level expression was occasionally detectable (1 heart without and 2 with late RA/TSA injection) 10 weeks after HD vector administration. These findings are consistent with a previous report showing early and complete loss of transgenic β-galactosidase expression, accompanied by predominantly $CD4^+$ and $CD8^+$ lymphocytic infiltrates and loss of vector DNA, after HD vector-mediated *LacZ* gene transfer into mouse skeletal muscle [6]. The same vector injected into transgenic mice constitutively expressing β-galactosidase achieved persistent expression of exogenous β-galactosidase, with no evidence of tissue inflammation. Furthermore, adenoviral expression of human erythropoietin was lost in mice, whereas exogenous mouse erythropoietin was expressed for extended periods of time [38]. Similar observations have been made using GFP as a reporter protein [37], indicating that immune responses to GFP or β-galactosidase can extinguish transgene expression. In the present study, similar peak GFP levels for the two vectors were observed at 1 week, but GFP levels at 2 weeks tended to be higher (not significant) with ΔE1 compared with HD vectors. Thus, the possibility that stronger immune

responses to higher GFP levels at 2 weeks induced more intensive tissue inflammation in the HD-vector group cannot be discarded. In apparent contrast to an immunogenic role for GFP, we previously reported long-term persistence of AAV-mediated GFP expression in mouse myocardium with no apparent inflammation [39]. These findings can be reconciled by experimental data showing that different vectors may induce distinct immune responses to the same transgenic protein. For instance, gene transfer of mouse coagulation factor IX in mice with hemophilia B induced cytoxic T lymphocytes to this factor using adenovirus, but not AAV, vectors [11]. A potential explanation is that adenovirus, unlike AAV, efficiently infects dendritic cells, which play a key role in antigen presentation, triggering cytoxic immune responses [21]. Also, several components of HD vectors including the transgene promoter and stuffer DNA affect their *in vivo* performance [33, 35]. For instance, a human CMV promoter, but not a phosphoglycerate kinase (PGK) promoter, within HD vectors resulted in an antibody response against human alpha-1 antitrypsin in mice [35]. Again, this observation was interpreted to mean that the generation of antibody responses against foreign antigens is induced by the use of a promoter that expresses the antigen in dendritic cells.

Recently, we and others reported on myocardial gene transfer using novel lentivirus vectors derived from human immunodeficiency virus-1 (HIV-1) [13, 43]. In our previous study [13], lentivirus vectors containing a CMV-driven GFP expression cassette were tested in the same experimental model used in the present study; hence, observations made in the previous study may be of interest for the interpretation of the present data. Using lentivirus vectors, we previously found a similar early decline in myocardial GFP expression compared with ΔE1 adenovirus vectors, despite the fact that lentiviral DNA is integrated into the host cell chromatin, whereas adenoviral DNA is not. Loss of integrated lentivirus DNA paralleled the early loss of GFP expression, supporting immune-mediated clearance of transduced cells as the underlying mechanism. This may also hold true for the progressive extinction of GFP expression with HD vectors, although myocardial inflammation observed with these vectors was milder than with lentiviral vectors (data not shown) [13]. Several factors including capsid toxicity, cell-derived proteins that co-purify with vector particles, helper virus particles in HD vector preparations, and needle injury may account for the residual, low-level tissue inflammation observed with HD vectors. The main difference between lentivirus and HD vectors was that lentiviral GFP expression stabilized after week 4 and was still detectable at week 10, whereas HD vector-mediated expression was only rarely detectable at this late time point, consistent with the intrinsic instability of episomal (i.e., non-integrated) adenovirus DNA.

Intracellular mechanisms may lead to specific shut off in gene expression or elimination of the vector genome without destruction of infected cells [33], as described for hepatitis B virus in chimpanzees [17]. Transcriptional silencing is a frequent cause of shut off in gene expression using constitutive viral promoters [9, 14]. The transcriptional activity of the human CMV promoter is regulated by several cytokines [31], some of which were induced, to different extents, by adenovectors in the present study. In an attempt to reactivate the CMV promoter within HD vectors, we injected RA and TSA [9, 14] into the heart in a small subgroup of rats 10 weeks after gene transfer. Four days later, GFP expression was detectable in 2 out of 3 hearts receiving RA/TSA, but in none of those injected with buffer solution at 10 weeks (Fig. 2C). This observation suggests, but does not prove, that transcriptional silencing may play a contributory role in loss of myocardial GFP expression with HD vectors.

Finally, we tested HD vectors in rat carotid arteries. Titer-matched HD and ΔE1 vectors were equally efficient at transducing vascular endothelium *in vivo*. However, numbers of endothelial cells expressing GFP at a histologically detectable level were relatively modest using the available vector concentrations. Differences in large-scale production techniques between HD and ΔE1 vectors prevented testing of more concentrated HD vectors in this model. Higher-titer HD vectors may be needed to further improve endothelial transduction *in vivo*.

From a clinical point of view, the preeminence of HD vectors over earlier generation adenovirus vectors should be considered within the framework of available clinical data. A recent report summarizing safety aspects of ΔE1/ΔE3 adenovirus vectors in several different gene therapy trials (n = 90 patients) showed no death directly related to the vectors, and a 0.7 % total incidence of major adverse events linked to them [19]. In three gene therapy trials for coronary artery disease, patients received up to 4×10^{10} particle units of intramyocardial ΔE1/ΔE3 adenovirus vector expressing vascular endothelial growth factor-121 (VEGF-121) [32], or up to 3.3×10^{10} TU of intracoronary ΔE1 vector expressing fibroblast growth factor-4 (FGF-4) [16]. In the VEGF-121 gene trial (phase-I) [32], there were 2 perioperative deaths unrelated to adenovirus administration. In one of them, autopsy revealed no abnormalities in the myocardial territory treated with the vector. No perioperative increase in myocardial enzymes, and no ECG or echocardiographic changes were observed in this trial. In the FGF-4 gene trial (phase-I), transient fever occurred in 3 patients receiving the highest dose of the vector, but no severe adverse events were related to it. In the FGF-4 gene phase-II trial [16], there were no deaths, myocardial infarction, or myocarditis, but transient fever and rise in serum bilirubin occurred in one patient in the adenovirus group. A tendency toward a functional improvement was observed in the treated group. Obviously, clin-

ical manifestations may not adequately reflect mild to moderate myocardial inflammation, and histological analysis was not feasible in most cases. Thus, despite clinical evidence that ΔE1 vectors were relatively well tolerated in cardiovascular trials, HD vectors may be safer for future applications.

In conclusion, HD vectors are as efficient as earlier generation adenovirus vectors in cardiovascular gene transfer models. However, they are substantially less toxic, at least under this study's experimental conditions. Moreover, HD vectors offer a large capacity to accommodate large DNA fragments, potentially allowing the simultaneous expression of several genes, genomic DNA, large cDNAs, and the introduction of large regulatory DNA regions. Recently, it has been shown that the use of genomic DNA may be advantageous over small cDNA expression cassettes for gene transfer-based strategies [35]. Thus, high-capacity adenovirus vectors may be useful tools in the armamentarium of cardiovascular gene therapy.

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