Adenoviral gene transfer to the heart during cardiopulmonary bypass: effect of myocardial protection technique on transgene expression

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Abstract

Objective: Adenoviral gene transfer to the arrested heart during cardiopulmonary bypass (CPB) is a novel method of allowing prolonged vector contact with the myocardium. In this model we investigated the importance of temperature, duration of arrest and cardioplegia on transgene expression. Methods: First-generation adenoviral vector (1 \times 10^{12} total viral particles) containing the transgene for the human \( \beta_2 \)-adrenergic receptor (Adeno-\( \beta_2 \)-AR) or \( \beta \)-galactosidase (Adeno-\( \beta \)-gal) was delivered to neonatal piglets via the proximal aorta, during simulated cardiac surgery, and allowed to dwell for the cross-clamp duration. Four treatment groups received Adeno-\( \beta_2 \)-AR. Groups A (n = 4) and B (n = 6) underwent cold crystalloid cardioplegia arrest for 10 and 30 min, respectively. Group C (n = 5) underwent warm crystalloid cardioplegia arrest for 10 min, and Group D (n = 5) underwent warm fibrillatory arrest for 10 min. Group E (n = 6) received Adeno-\( \beta \)-gal and underwent cold crystalloid cardioplegia arrest (30 min). Animals were weaned off CPB and recovered for 2 days. Receptor density was assessed in membrane fractions using radioligand binding and compared using the Mann-Whitney \( U \)-test. Results: Left ventricular transgene overexpression, as evidenced by elevated \( \beta \)-AR density, following Adeno-\( \beta_2 \)-AR treatment was greatest with cold cardioplegia (Group A 588 \pm 288.8 fmol/mg; \( P = 0.002 \)) versus control (Group E 109 \pm 8.4 fmol/mg). Overexpression also occurred with warm cardioplegia (Group C 274 \pm 69.5 fmol/mg; \( P = 0.05 \)) and ventricular fibrillation (Group D 215 \pm 48.4 fmol/mg; \( P = 0.02 \)). Comparison of the combined cold cardioplegia groups versus those treated with warm conditions showed a trend towards increased expression with cold conditions (\( P = 0.1 \)). Receptor density was also significantly increased in the right ventricle of animals in Group B (165 \pm 18.1 fmol/mg; \( P = 0.03 \)) and Group D (181 \pm 23.4 fmol/mg; \( P = 0.02 \)) versus control (Group E 118 \pm 5.8 fmol/mg). Conclusions: Cold crystalloid cardioplegia is not detrimental to gene transfer in vivo. In fact, there was a trend towards increased left ventricular transgene expression when the adenoviral vector was delivered following cold versus warm cardioplegia. Shorter periods of contact with the vector may reduce transgene overexpression. Therefore, gene transfer is possible during cardiac surgery with clinically used myocardial protection techniques. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Gene therapy to modify the \( \beta \)-adrenergic receptor signalling pathway has been proposed as a possible treatment for heart failure [1]. Existing transgenic mice models have demonstrated improved cardiac function following myocardial specific overexpression of the \( \beta_2 \) adrenergic receptor (\( \beta_2 \)-AR) or expression of an inhibitor of \( \beta \) adrenergic receptor kinase, the carboxyl terminus of \( \beta \) adrenergic receptor kinase, \( \beta \)ARKct [2,3]. However, transgenic models differ greatly from gene transfer models. Many existing methods of vector delivery have limited clinical applicability. Direct myocardial injection has confirmed the possibility of gene transfer to the myocardium [4]. Intracoronary delivery in rabbits has been performed during thoracotomy, with the aorta cross-clamped and the virus introduced by injection into the left ventricular cavity [5–7]. Due to the occluded aorta, left ventricular ejection forces the virus down the coronary arteries. However, the risk of ischaemia and acute pressure overload of the left ventricle limit the period of cross-clamping. A more clinically applicable method is direct catheterization of the right or left coronary artery [8]. Here too,
exposure of the heart to the virus is limited as the adenovirus is rapidly washed out into the systemic circulation and other tissues have shown evidence of transgene expression [5,6,9].

By means of a larger animal model we have developed a method of gene delivery to the myocardium that could be more clinically applicable. During standard cardiopulmonary bypass (CPB) the heart was arrested, the aorta cross-clamped and the adenoviral vector administered after the cold cardioplegia. Global cardiac transgene expression was obtained with no evidence of extracardiac expression [10].

In a Langendorff perfused rabbit heart model it has been demonstrated that temperature, duration of exposure to vector and the solution in which the vector is delivered can all influence cardiac transgene overexpression [11]. Therefore, the current study was undertaken to investigate the effect which these factors have on adenoviral based gene transfer in the cardiac surgery model.

2. Material and methods

2.1. Adenoviral transgenes

The adenoviral backbone used was a replication-deficient first generation type V adenovirus with deletions of the E1 and E3 genes. Two vectors were constructed containing either the human β2-AR transgene (Adeno-β2AR) or a cytoplasmic expressing β-galactosidase transgene (Adeno-βgal). Large-scale preparations of these adenoviruses were purified from infected Epstein–Barr nuclear antigen (EBNA)-transfected 293 cells (Invitrogen Corp., CA) as previously described [12]. Immediately prior to use the adenoviral vector was thawed from −80 °C and reconstituted in 8 ml phosphate-buffered saline (PBS; Dulbecco Phosphate Buffered Saline, Gibco BRL, Life Technologies, Grand Island, NY).

2.2. Animals

Twenty-six neonatal piglets (1–2 weeks old), weighing approximately 3 kg were used in these studies. Six-month old pigs weighing up to 40 kg were killed to provide fresh blood for priming of the CPB circuit. All animals received humane care in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication No. 85-23, revised in 1985). The experimental protocol was approved by the institutional Animal Care and Use Committee.

2.3. Surgery, cardiopulmonary bypass and gene delivery

Animals were anesthetized and CPB was established as previously described [10]. After stabilization, the aorta was cross-clamped and the heart arrested by infusion of cardioplegia into the aortic root or by induction of ventricular fibrillation. The cardioplegia consisted of lactated Ringer’s solution with 40 mmol/l KCl added (Abbott Laboratories, North Chicago, IL). A volume of 30 ml/kg cardioplegia was injected via a hand-held syringe at a rate of approximately 50 ml/min through a 21-gauge butterfly cannula (Abbott Laboratories) inserted into the proximal aorta. Immediately following delivery of cardioplegia, or induction of fibrillatory arrest, the adenoviral vector, in 8 ml PBS, was injected through the cardioplegia cannula over a 10-s period. The catheter and proximal aorta were flushed with a further 2 ml of PBS. Saline slush was placed within the pericardial cavity to provide topical cooling and aid myocardial protection for those groups receiving cold myocardial protection techniques.

In order to test the effect of temperature, duration of arrest and type of myocardial protection on transgene expression, a total of four test groups (Adeno-β2AR; Groups A–D) were employed and one control group (Adeno-βgal; Group E). For each group, 1 × 10¹² total viral particles (tvp) were administered into the aortic root following cross-clamping of the aorta and arrest of the heart. Specifically, Group A (n = 4) underwent cold crystalloid cardioplegia arrest for 10 min, Group B (n = 6) underwent cold crystalloid cardioplegia arrest for 30 min, Group C (n = 5) underwent warm crystalloid cardioplegia arrest for 10 min, and Group D (n = 5) underwent warm fibrillatory arrest for 10 min. Group E (n = 6) was treated with Adeno-βgal following cold crystalloid cardioplegia arrest (30 min).

Therefore, the vector was allowed to dwell in the coronary circulation for 10 min in Groups A, C and D prior to release of the aortic cross-clamp. In Groups B and E a 30-min dwell-time was allowed before the cross-clamp was released. The heart was defibrillated as required, the animals were weaned off CPB and allowed to recover.

2.4. Collection of tissue samples

Two days following surgery, euthanasia was performed, using an intravenous injection of 16 mEq potassium chloride solution. The chest was reopened and the heart rapidly excised.

2.5. β-Galactosidase staining

After excision of the heart, transverse cross-sections of myocardium at the level of the base of the papillary muscle were obtained. Frozen samples were mounted on a cryostat and 10-μm sections were cut, which were then mounted on a glass microscope slide. β-Galactosidase staining was performed in 20 mM K₄Fe₆(CN)₆, 20 mM K₃Fe₆(CN)₆, 4 mM MgCl₂, 0.5 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside) in 1 M PBS at 37 °C for 90 min [6].
2.6. Preparation of myocardial membranes

Tissue samples from the anterior left ventricle (LV) and right ventricle (RV) were homogenized in lysis buffer (5 mM Tris–HCl (pH 7.4), 5 mM EDTA). Membrane fractions were resuspended in binding buffer (75 mM Tris–HCl (pH 7.4), 12.5 mM MgCl2, and 2 mM EDTA) at a concentration of approximately 1 mg/ml of membrane protein as determined by the Bradford method [2,13].

2.7. Ligand binding assay

Ligand binding assays were performed in triplicate on membranes with saturating concentrations of the radiolabelled β-adrenoceptor ligand, [125I]cyanopindolol. Non-specific binding was determined in the presence of alprenolol (20 μM). Assays were performed at 37 °C for 60 min [2,6]. The resulting specific binding (estimated Bmax) was reported as the receptor density (femtomoles) and was normalized to milligrams of membrane protein (fmol/mg membrane protein).

2.8. Statistics

Values are reported as mean ± standard error of the mean (SEM). Comparisons of receptor density between groups were performed using the non-parametric Mann–Whitney U-test.

3. Results

3.1. Adenoviral transgene delivery during cardiopulmonary bypass

As demonstrated in Fig. 1, global myocardial β-galactosidase expression at 2 days is evident by the X-Gal staining of hearts from animals in Group E which received Adeno-βgal. Sections of hearts treated with Adeno-β2AR revealed no X-Gal staining.

3.2. Adenoviral-mediated myocardial βAR over-expression

Left ventricular over-expression of β2AR was evidenced by elevated βAR receptor density in the LV of animals in Groups A, B, C and D compared with the control, Group E (Fig. 2). The highest level of overexpression occurred in the animals that received cold cardioplegia (Group A 588 ± 288.8 fmol/mg membrane protein; P = 0.002 and Group B 520 ± 250.9 fmol/mg membrane protein; P = 0.01) compared with control (Group E 109 ± 8.4 fmol/mg membrane protein). Receptor density for animals treated with warm cardioplegia (Group C 274 ± 69.5 fmol/mg membrane protein; P = 0.05) and for animals treated with warm fibrillatory arrest (Group D 215 ± 48.4 fmol/mg membrane protein; P = 0.02) were also significantly
increased relative to control. Comparison of the combined groups of animals treated with cold cardioplegia (Groups A and B, \( n = 10; 548 \pm 179.5 \) fmol/mg membrane protein) versus those treated with warm conditions (Groups C and D, \( n = 10; 244 \pm 41.1 \) fmol/mg membrane protein) showed a trend towards increased expression with cold conditions (\( P = 0.1 \)).

Receptor density was significantly increased in the RV of animals in Group B which had a 30-min cardiac arrest with cold cardioplegia (165 ± 18.1 fmol/mg membrane protein; \( P = 0.03 \)) as shown in Fig. 3. However, Group A which also had cold cardioplegia but only a 10-min cross-clamp period showed no increase in RV receptor density (118 ± 2.2 fmol/mg membrane protein; \( P > 0.2 \)). Similarly, Group C which received warm cardioplegia with a 10-min cross-clamp showed a non-significant increase in receptor density (131 ± 16.8 fmol/mg membrane protein; \( P > 0.2 \)). In contrast, Group D which had warm conditions for 10 min with ventricular fibrillation had the highest RV receptor density (181 ± 23.4 fmol/mg membrane protein; \( P = 0.02 \)).

4. Discussion

This study demonstrates that increased \( \beta \)AR density occurs following Adeno-\( \beta \)AR delivery to the arrested heart of neonatal piglets during CPB. LV \( \beta \)AR density was increased in all groups compared with Adeno-\( \beta \)gal controls, although the highest receptor densities occurred in the groups which received cold crystalloid cardioplegia. \( \beta \)AR density was lower in the RV relative to the LV, but was significantly increased over control for two of the Adeno-\( \beta \)AR-treated groups.

The optimal conditions for adenoviral based gene transfer to cultured rabbit ventricular myocytes in vitro have been investigated [11]. Delivery of Adeno-\( \beta \)gal at 37 °C caused infection of approximately 50% of myocytes after 10 s exposure but this rose to 100% infection following 10 min of contact. When incubated at 37 °C the time to half-maximal infection was 19 min. Reduction of the temperature to 24 °C prolonged the time to half-maximal infection with no change in the final peak but when the temperature was reduced to 4 °C both the time to half-maximal infection and the final peak were reduced. The presence of heparinized rabbit blood caused a 60% reduction in the number of infected cells compared with incubating the myocytes and virus solution in M199 media or Krebs buffer. The optimal conditions for adenoviral based gene transfer were investigated further in a Langendorff perfused rabbit heart. A single pass of the virus solution containing \( 5 \times 10^9 \) plaque forming units through the heart caused infection of 0.8% myocytes in contrast to 40% infection when the virus perfusate was recirculated for 60 min.

In view of these findings it was surprising that in an in vivo delivery system temperature did not seem to be important for improving gene transfer. LV \( \beta \)AR density was actually lower for those groups that had warm conditions during the period that the virus solution was allowed to dwell in the coronary circulation, namely warm crystalloid cardioplegia and ventricular fibrillation, compared with a similar 10-min cross-clamp period following cold crystalloid cardioplegia. The absence of a significant influence of cold temperatures on transgene expression in an in vitro model may be due to the fact that attachment of adenovirus to cells occurs efficiently at 0 °C [14]. Furthermore, it is possible that while the viral vector is delivered to the interstitium under cold conditions it may only be taken up into myocytes after warm reperfusion. Myocardial temperature was not monitored in these experiments but it is clear that myocardial temperatures will quickly return towards the systemic body temperature of 37 °C following the return of coronary blood flow, after release of the aortic cross-clamp. Thus, while subsequent steps in adenoviral infection are inhibited at low temperatures, it seems that the temperature during the first 30 min does not limit infection [15].

The period of cross-clamping following adenoviral vector
delivery may influence transgene expression. There was similar LV βAR density in the group which had a 10-min cross-clamp period compared with the 30-min cross-clamp period after cold crystalloid cardiotomy. However, RV βAR density was not increased in the 10-min cross-clamp period compared with the Adeno-βgal group while the 30-min cross-clamp group did have increased RV βAR density. In general, transgene expression in the RV was considerably lower relative to the LV. We have previously seen this pattern of reduced RV overexpression in this model [10]. For unclear reasons, RV delivery of the vector is impaired, or conversely wash-out of the vector from the RV is increased, relative to the LV.

The vector was resuspended in a crystalloid solution, namely PBS, in all groups. This was because adenovirus can agglutinate red blood cells and so potentially reduce the number of viral particles available for transfer through the endothelium and subsequent cell entry [14]. The additional presence of crystalloid cardioplegia compared with blood within the coronary circulation, as in the fibrillatory arrest group, had no discernable effect on transgene expression.

In all these cases the virus solution was administered in a bolus injection. Continuous circulation of an adenoviral vector solution through the heart has been suggested to be another potential method of gene delivery during CPB, although this requires the use of two separate circuits and oxygenators, unlike the standard approach [16]. Gene transfer has been documented using retrograde delivery of vector through the coronary sinuses in a porcine beating heart model [17]. This is a further method that could also be utilized for vector delivery during cardiac surgery.

Additional work in the Langendorff perfused rabbit heart suggested that agents which increase endothelial permeability can greatly enhance the efficiency of gene transfer [18]. Cardioplegia has detrimental effects on endothelial structure and function [19,20]. Further experiments could be performed to assess if gene transfer is enhanced when the virus solution is delivered to the heart following a prolonged period of cardioplegic arrest. Contact with cardioplegia and the associated relative ischemia may increase endothelial permeability, thus overcoming one of the barriers to adeno-viral infection of cardiac myocytes. Furthermore, if delivery of a gene that could enhance postoperative cardiac function was possible during CPB, this might be especially desirable towards the end of a prolonged cross-clamp period when impaired cardiac function was manifest. Although transgene expression was assessed 2 days after vector delivery in this study, we have previously documented that transgene expression is present 8 h after vector delivery [10]. Over-expression of the β2AR has been shown to have functional consequences following gene transfer in other animal models [6,8]. This method of vector delivery could, therefore, be used to counteract postoperative ventricular dysfunction which can complicate cardiac surgical recovery [21].

This series of experiments demonstrate that in an in vivo model of simulated cardiac surgery, adenoviral based gene transfer is not impaired by the lower temperatures associated with cold crystalloid cardioplegic arrest. Shorter durations of exposure to the vector may play a limited role in the final level of transgene overexpression. The presence of blood versus crystalloid solution within the heart does not have a major effect. Thus, adenoviral based cardiac gene delivery is possible using various clinically utilized methods of myocardial protection in a cardiac surgical model.

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