Efficacy of adenoviral gene transfer with manganese superoxide dismutase and endothelial nitric oxide synthase in reducing ischemia and reperfusion injury


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Abstract

Objective: Both superoxide dismutase (SOD), a free radical scavenger, and nitric oxide (NO), a vasodilator with anti-inflammatory properties, have been shown to protect the myocardium from reperfusion injury. They are known to interact in vivo, the influence of which on myocardial protection has not been studied.

Methods: Four groups of rats (n = 7, per group) were subjected to experimental infarction following injections into the anterior wall of the left ventricle with adenoviral vector encoding β-galactosidase (group A), eNOS (group B), Mn-SOD (group C) and both eNOS and MnSOD (group D). Hearts were assessed for protein expression and size of infarction.

Results: Efficiency of gene up regulation was confirmed by immunostaining for eNOS and Mn-SOD, and X-gal staining for β-gal respectively. In B and D, overexpression of eNOS was demonstrated in cardiac myocytes in addition to that in the endothelium, while in C and D, Mn-SOD was overexpressed in mainly cardiomyocytes. Infarct size was 49.7 ± 4.8% in A, and was significantly reduced in the other groups (29.8 ± 2.7%, 21.8 ± 2.5% and 24.9 ± 2.4% in B, C and D respectively).

Conclusion: Adenoviral gene transfer of Mn-SOD was superior to eNOS in reducing the extent of in vivo ischemia-reperfusion injury in the rat heart in our model. The effect of combined application of Mn-SOD and eNOS was not different from their individual effect.

Keywords: Adenovirus; Gene transfer; Myocardial infarct, Ischemia/reperfusion; Nitric oxide synthase; Superoxide dismutase

1. Introduction

The production of oxygen free radicals is an important factor involved in myocardial ischemia-reperfusion injury [1,2]. Superoxide dismutase (SOD) catalyses the dismutation of superoxide anion (O$_2^-$) to oxygen and hydrogen peroxide [3]. Manganese SOD (Mn-SOD), an isoform found in the mitochondrial matrix, is induced by high oxygen tensions and by compounds, which cause the production of intracellular O$_2^-$ [4]. It has been demonstrated that the infarct limitation after ischemic preconditioning, is accompanied by a significant increase in the activity of Mn-SOD [5]. Moreover, transgenic mice overexpressing MnSOD seem to be more resistant to myocardial ischemia-reperfusion [6].

The endothelium produces nitric oxide (NO) [7], which is a potent vasodilator [8] and exerts anti-neutrophil actions that reduce the inflammatory components of ischemia-reperfusion injury [9]. Studies involving experimental coronary artery occlusion and reperfusion suggested that early damage to the coronary endothelium impairs NO production. It has been shown that NO donors can reduce the infarct size in models of coronary occlusion and reperfusion [10]. It has also been shown that localised gene transfection with endothelial nitric oxide synthase (eNOS) is an efficient method for reducing luminal narrowing after coronary angioplasty in pigs [11]. On the other hand, NO is reported to react with the superoxide anion to form peroxynitrite [12] which in turn exacerbates the deleterious effects of free radicals. Moreover, superoxide radicals can inactivate NO [13], such that during reperfusion, neutrophil aggregation and adherence is enhanced. These interactions made a strong case for attempting to overexpress both eNOS and MnSOD, together, in the endothelium and cardiomyocytes respectively, which to our knowledge has not been studied before. In this study, we examined the effect of
adenoviral-mediated gene transfer of recombinant Mn-SOD and eNOS alone or in combination on infarct size following ligation of the left main coronary artery in a rat heart model.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (Weight 280–300 g) were used in this study. All animals received humane care in compliance with the ‘Principles of Laboratory Animal Care’, formulated by the National Society for Medical Research; the ‘Guide for the Care and Use of Laboratory Animals’, prepared by the Institute of Laboratory Animal Resources (NIH publication No. 86-23, revised 1985); and the ‘European Convention on Animal Care’ guide. The study has been approved by the institutional ethics committee on animal research.

2.2. Adenoviral vector

A serotype 5 adenovirus encoding for non-nuclear targeted Escherichia coli β-galactosidase under the control of the cytomegalovirus promotor was used in the control group (AdCMVLaCZ, provided by James Wilson, Institute for Gene Therapy, University of Pennsylvania, PA, USA). This vector has been rendered replication defective by replacing the entire Ela and most of the E1b regions of the adenoviral genome with the complementary DNA expression cassette. Adenoviral vector encoding eNOS was generated as previously described [14]. In brief, bovine eNOS cDNA was cloned into the shuttle plasmid pACCMVVpLpA. The resulting plasmid was linearized with NruI and cotransfected with d1309 into 293 cells by calcium phosphate/DNA coprecipitation. D1309 is a biologically selected, restriction enzyme-site-loss variant of wild type adenovirus type 5, which retains only a single Xba1 site at nucleotide 1339. Two hundred and ninety-three cells are human embryonic kidney carcinoma cells that have been transformed with the left end of human adenovirus type 5 DNA. Recombinant adenovirus vectors were generated by homologous recombination. Viral plaques were picked and propagated in 293 cells. Viral DNA was enriched by Hirt extraction and screened by restriction mapping and polymerase chain reaction (PCR) for the presence of eNOS cDNA. Positive plaques underwent two further rounds of plaque purification in 293 cells. Stocks were prepared from positive plaques and these were used to generate high titer preparations. Viral preparations were prepared by infecting a confluent monolayer of 293 cells in T175 flasks with viral stock at a multiplicity of infection (MOI) of 1–10. Virus was purified by double cesium gradient ultracentrifugation and was dialyzed against 10 mmol/l Tris, 1.0 mmol/l MgCl2, 1.0 mmol/l HEPES and 10% glycerol for 4 h at 4°C. Viral titer was determined by plaque assay. MnSOD recombinant adenoviral construct was generated using a previously described method. Briefly, MnSOD constructs were generated by cloning of an EcoRI/PvuII fragment from the pRK5 MnSOD construct [15]. Recombinant adenoviral plasmid construct were generated by cloning transgene into pAd.CMVlink, which contains the CMV enhancer/promotor and an SV40 polyadenylation site for efficient expression of the transgene [16]. Recombinant virus was generated by cotransfection of NheI-cut pAd plasmid with ClaI-cut Ad5.sub360 (E3-deleted) viral DNA [17]. After transfection, plates were overlaid with agar, and initial plaques were harvested for screening by enzymatic activity. This recombinant virus was screened for MnSOD activity by secondary infection on 293 cells. Initial plaques that expressed functional enzyme were further purified through two subsequent rounds of plaque purification. Viral titer was determined by assessing pfu on 293 cells.

2.3. Experimental groups and outlined protocol

Rats were divided into four groups according to the gene encoded by the adenoviral vector; group A (LaCZ, n = 7) as control, group B (eNOS, n = 7), group C (Mn-SOD, n = 7) and group D (eNOS + Mn-SOD, n = 7). Hearts were regionally transfected with the vectors. Four days later, allowing for gene expression, all hearts were subjected to left main coronary artery occlusion and reperfusion (Fig. 1) followed by an assessment of infarct size using planimetry as described below.

2.4. Myocardial gene transfer

Animals were anaesthetised with sodium pentobarbital (30 mg/kg, intraperitoneally), intubated through the endotracheal route, and ventilated with room air using a Harvard Rodent Ventilator with a volume of approximately 1.2 ml/100 g body weight and a rate of 68–70 strokes/min. Under a sterile technique the chest was opened through the left fourth intercostal space to expose the beating heart, the pericardium was stripped and the left main artery was identified at the junction between the left atrium and the pulmonary artery. The area of myocardium around the artery was directly injected with a total adenoviral dose of 1.0 × 109 plaque forming units (pfu/ml, delivered in six injections (25 μl of 0.16 × 109 pfu/ml each) using a 50 μl syringe with a 25 G needle. In group D hearts, the total dose of the virus delivered was 2.0 × 109 pfu/ml (25 μl of 1.0 × 109 pfu/ml of eNOS and 25 μl of 1.0 × 109 pfu/ml of Mn-SOD). Chest air was expelled, surgical incision was closed in layers. Animals were left to recover on the ventilator with oxygen in a warm environment.

2.5. Regional ischemia-reperfusion

On the fourth day after gene transfer, animals were re-anaesthetised, ventilated and the chest re-opened. A 6-0 polypropylene suture (Prolene; Ethicon) was passed loosely around the left main artery near its origin. Coronary occlu-
sion was performed by tightening the suture for 30 min. Acute myocardial ischemia was deemed successful on the basis of regional cyanosis of the myocardial surface distal to the suture. The loop was then loosened and the ischemic myocardial area reperfused for 2 h, as identified on the basis of return of the original colour. At the end of the reperfusion period, animals were heparinised (200 IU) intravenously, sacrificed using an overdose of pentobarbital and the heart arrested with cold (4°C) crystalloid (St. Thomas’ No. 1) cardioplegia, injected through the abdominal aorta and harvested. The coronary snare was left in-situ to allow for infarct size studies.

2.6. Infarct size assessment

The excised heart was cannulated through the aorta (using a 1.2 x 45 mm cannula), and flushed with normal saline to remove any remaining blood clots. The coronary snare was tightened and the heart perfused through the aortic cannula with saline containing 4% Monastral blue dye to determine the area at risk of infarction (area which does not stain). The atria, great vessels and right ventricle were dissected. The left ventricle was kept at 5°C for 24 h and then cut into four slices transversely from the base to apex. The slices were incubated at 37°C in saline containing 4% triphenyltetrazolium for 30 min, to stain non-infarcted tissue. The stained sections were placed in 10% formaldehyde and 24 h later placed caudal side up between glass plates with 2 mm spaces. Area at risk of infarction (area without Monastral stain) and area of infarction (area without triphenyltetrazolium) were traced on transparent paper and downloaded into the computer. Areas were measured with the use of NIH Image program. The following parameters were averaged for four slices from each heart: (1) infarct size expressed as a percentage of the area at risk and (2) area at risk expressed as a percentage of the total area of the slice.

2.7. Assessment of gene transfer efficiency

Samples were taken from around the injection sites and then frozen in liquid nitrogen for immunostaining analysis using X-gal or antibodies specific for eNOS or Mn-SOD. Group A hearts were bisected, embedded in OCT medium (Miles, Elkhart, IN, USA) and frozen in liquid nitrogen. Frozen sections (6 μm thick) were fixed in 2% paraformaldehyde, 0.125% glutaraldehyde in PBS for 5 min, washed three times in PBS with 2 mM magnesium chloride then incubated in three changes of PBS containing 2 mM magnesium chloride, 0.01% sodium deoxycholate and 0.02% NP-40. Sections were then incubated in staining buffer (30 mM potassium ferrocyanide and 30 mM potassium ferricyanide in PBS containing 2 mM magnesium chloride, 0.01% sodium deoxycholate and 0.02% NP-40) for 2 min prior to incubation in fresh staining buffer containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal) and incubated in a moist chamber overnight at 37°C. Subsequently, sections were rinsed in PBS and counterstained with neutral red and rinsed in water before mounting. Blue-stained cells indicated the presence of β-galactosidase expression. Immunohistochemical staining for eNOS or Mn-SOD was performed with heart specimens from around the injection sites. Samples were embedded in OCT medium (Miles, Elkhart, IN, USA) and frozen in liquid nitrogen. Frozen sections (5 μm thick) were cut at 25 μm intervals, fixed for 10 min in cold acetone (4°C), fan-dried for 10 min and further fixed in 1% paraformaldehyde/EDTA for 3 min. Endogenous peroxidase activity was blocked with 0.1% sodium azide/0.3% H2O2 for 10 min. Incubating sections with 5% goat serum/PBS-Tween 20 blocked non-specific protein binding sites. Samples from Group A, B and D then had (1:250) of anti-eNOS monoclonal antibody (N30020) (Transduction Laboratories, Lexington, KY, USA) added and were incubated for 60 min at room temperature. After rinsing, biotinylated rabbit anti-mouse F(ab’)2 (1:300) was added for 20 min. After further incubation for 20 min with peroxidase conjugated-streptavidin (1:300), the slides were incubated for 30 s in 0.1 M sodium acetate buffer, pH 5.2. Then, were placed in 3-amino-9-ethylcarbazole substrate solution and incubated for 15 min at room temperature, counterstained in mercury-free hematoxylin for 1 min and further rinsed for 3 min in cold running tap water before being mounted. For Mn-SOD staining in group A, C and D samples, a similar protocol as above was
used, with the primary antibody (K90096C) from BioDesign, UK, at a dilution of 1:200. The secondary antibody was 1:1000 of sheep/goat peroxidase (M15345).

2.8. Statistics

Values are presented as means ± standard error of the mean (SEM). Analysis of variance (ANOVA) followed by Bonferroni test to indicate individual significant differences. A value of $P < 0.05$ was considered as a significant difference. Infarct size was expressed as a percentage of the area at risk.

3. Results

The efficiency of the adenoviral vector in transfecting rat cardiomyocytes was confirmed in the LacZ transfected group. Positive myocardial staining with X-gal can be seen in many myocytes while this has not been observed in controls (Fig. 2). Immunohistochemical staining for eNOS in groups A and B showed weak endothelial expression of native eNOS in group A and both endothelial and cardiomyocyte expression of eNOS in group B (Fig. 3). It shows some distortion of myocardial architecture (needle tract injury) and a number of inflammatory cells (immunological reaction to the adenoviral vector). Fig. 4 presents immunohistochemical analysis of Mn-SOD expression in groups A and C, showing overexpression of Mn-SOD in the group C hearts in many myocytes. Group A hearts showed weak staining for native Mn-SOD in cardiac myocytes. Distorted architecture and inflammatory cells were found in these slides. Group D hearts were analysed for eNOS and Mn-SOD separately; overexpression of each gene was detected in each sample tested, Fig. 5. It showed overexpression of eNOS in mainly endothelial cells and a smaller expression in myocytes. Over expression of Mn-SOD was seen in cardiomyocytes. Myocardial damage and inflammation were seen in these samples.

Fig. 6 shows staining done with both; 4% Monastral blue dye to determine the area at risk of infarction (AR, area which does not stain), and 1% triphenyltetrazolium to stain non-infarcted tissue, leaving the area of infarction (AI) without a triphenyltetrazolium stain. AI as a percentage of AR was calculated using planimetry. Area of infarction/area at risk ratio in group A hearts was almost twice as big as those in groups B, C and D (49.7 ± 4.8 vs. 29.8 ± 2.7, 21.8 ± 2.5 and 24.9 ± 2.4% in B, C and D respectively, $P < 0.05$), Fig. 7. Furthermore, infarct size was signifi-
4. Discussion

This study demonstrated a reduction in the area of infarction in hearts transfected with either Mn-SOD or eNOS genes as compared to the LacZ transfected hearts. Hence, each of these genes individually protected the heart against ischemia-reperfusion injury. On the other hand, there was no evidence for enhanced protection offered by the combined transfection with both of these genes. We have demonstrated that Mn-SOD gene may be superior in reducing the extent of ischemia-reperfusion injury in the rat heart, when compared to eNOS or the combination of eNOS and Mn-SOD.

Adenovirus has been demonstrated to be highly efficient in transgene expression in vivo through the direct intramyocardial injection method [18]. On the other hand, it was evident that the extent of transfection is limited to a small area around the injection site [18]. We showed in our experiment, that this restricted gene overexpression can provide the heart with added protection.

Although our results did not demonstrate that combined overexpression of eNOS and SOD is additive despite possible benefits, we have to interpret these results with caution. One possible problem is the use of higher virus dose in double transfected hearts. Adenovirus as a vector is known to trigger an inflammatory reaction, which may have been increased in double transfected hearts. Although, a number of inflammatory cells were seen on histological examination of single and double transfected groups, these hearts seemed not markedly different. Future work is needed with the same vector carrying both genes (Mn-SOD and eNOS) together.

Our study confirmed the efficiency of Mn-SOD in protecting the myocardium against ischemia-reperfusion injury. This finding agrees with the majority of studies on the cardioprotective effects of overexpression of this enzyme or its exogenous administration. There are three isoforms of SOD; copper/zinc SOD (Cu/Zn-SOD) which has a cytoplasmic location, extracellular SOD (EC-SOD) found in a variety of extracellular compartments and Manganese SOD (Mn-SOD) which is found in the mitochondrial matrix [19,20]. Jolly and associates [21] found that administration of SOD with catalase resulted in reduction of myocardial infarct size, in a canine model of ischemia-reperfusion. Recently, it was shown that in vivo adeno viral gene transfer of EC-SOD alone provides the heart with substantial protection against myocardial stunning [22]. We showed that in vivo gene transfer of Mn-SOD is an efficient method for enhancing antioxidant protection for cardiomyocytes and other cardiac cells.

Our experiment suggests that enhanced enzymatic activity of eNOS, can help ameliorate the effects of ischemia-reperfusion in the myocardium. We have demonstrated overexpression of this gene in the cardiac endothelium. On the other hand, in this study, eNOS was found to be less effective than Mn-SOD in achieving this goal. The...
transient nature of the eNOS-derived NO production is most beneficial during the early phase of reperfusion, which can explain part of our results. In addition to that, NO interacts with $O_2^-$ to form peroxynitrite [12], which in turn exacerbates the deleterious effects of free radicals, and further reduce the ability of NO to ameliorate ischemia-reperfusion injury. Further study is needed to test this finding in a different protocol of myocardial ischemia-reperfusion injury.

Our study did not investigate the mechanisms of protection offered by Mn-SOD or eNOS gene overexpression. Furthermore, since these genes were overexpressed before, during and after ischemia, it would not be possible to determine with certainty whether ischemic or reperfusion injury was the target for this protective effect. However, Mn-SOD mediated protection is related to free radical damage, which is known to occur during reperfusion. It is thus more likely that the reperfusion phase was a major target for protection. Further studies using enzyme inhibitors may establish a critical phase of protection offered by overexpression of either of the genes studied.

Although clear benefits of Mn-SOD an eNOS were demonstrated here, much work needs to be done before safe gene transfer technology will be developed for clinical application. However, the principle of increasing activity of Mn-SOD and eNOS before cardiac ischemia remains an attractive therapeutic strategy in cardiac surgery for high-risk patients or those with unstable angina.

5. Conclusions

The combined gene transfer of recombinant Mn-SOD and eNOS has no additive protective effect compared to single gene transfer in attenuating myocardial damage under conditions of experimental myocardial infarction. Mn-SOD offered the most significant protection in this setting.

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