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S100A1 Gene Transfer in Myocardium

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Abstract
S100A1, a Ca^{2+}-binding protein of the EF-hand type, is preferentially expressed in myocardial tissue and has been shown to enhance cardiac contractile performance by regulating both sarcoplasmic reticulum (SR) Ca^{2+}-handling and myofibrillar Ca^{2+}-responsiveness. In cardiac disease, the expression of S100A1 is dynamically altered as it is significantly down-regulated in end stage human heart failure (HF), and it is up-regulated in compensated hypertrophy. Therefore, the delivery of a transgene encoding for S100A1 to the myocardium might be an attractive strategy for improving cardiac function in HF by replacing lost endogenous S100A1. In this study we sought to test whether exogenous S100A1 gene delivery to alter global cardiac function is feasible in the normal rabbit heart.

An adenoviral S100A1 transgene (AdvS100A1) also containing the green fluorescent protein (GFP) was delivered using an intracoronary injection method with a dose of 5×10^{11} total virus particles (tvp) (n = 8). Rabbits treated with either a GFP-only adenovirus (AdvGFP) or saline were used as control groups (n = 11 each). Seven days after global myocardial *in vivo* gene delivery hemodynamic parameters were assessed. S100A1 overexpression as a result of the intracoronary delivery of AdvS100A1 significantly increased left ventricular (LV) $+dP/dt_{max}$, $-dP/dt_{min}$ and systolic ejection pressure (SEP) compared to both control groups after administration of isoproterenol (0.1, 0.5 and 1.0 μg/kgBW/min), while contractile parameters remained unchanged under basal conditions. These results demonstrate that global myocardial *in vivo* gene delivery is possible and that myocardial S100A1 overexpression can increase cardiac performance. Therefore, substitution of down-regulated S100A1 protein expression levels may represent a potential therapeutic strategy for improving the cardiac performance of the failing heart.

Key words: S100A1, gene therapy, contractile function, myocardium

Introduction

Heart failure (HF) continues to represent an enormous clinical challenge, since the mortality of patients with end stage heart failure is still similar to patients suffering from neoplastic diseases and projections are that the incidence of HF in the coming years will rise significantly [1,2]. Therefore, novel therapeutic approaches to support stressed myocardium and thus to prevent progressive deterioration of ventricular function are of great interest.

It is widely accepted that the dysfunction of the sarcoplasmic reticulum (SR) in the failing heart contributes to the reduced cardiac function in HF. Decreases in SR Ca^{2+} release and ryanodine receptor (RyR2) expression have been observed in human HF [3]. SR Ca^{2+}-ATPase (SERCA2a) levels are decreased in the failing heart leading to impaired SR uptake of Ca^{2+} [4]. Moreover, NCX levels have been shown to be increased in HF, which can contribute to lower intracellular Ca^{2+} in this disease [4], and SERCA2a expression as well as the phosphorylation status of its regulator Phospholamban (PLB) are both lower in HF, which can alter SR function [4, 5]. Overall, cardiac SR function and Ca^{2+} signalling is significantly deranged and increased cytosolic Ca^{2+} levels during diastole and decreased systolic Ca^{2+} peaks contribute to the compromised diastolic relaxation and systolic contraction of the failing heart [6].

S100A1, an EF-hand type Ca^{2+}-binding and sensing protein, is preferentially expressed in healthy myocardium [7, 8] being predominantly localized with the SR and with actin stress fibers. In cardiac disease, the expression of S100A1 is altered as it is significantly down-regulated in end stage human HF [8], and it is up-regulated in compensated hypertrophy [9]. Therefore, S100A1 might offer a therapeutical strategy to treat HF since S100A1 increased SERCA2a activity and exhibited a biphasic effect on the open probability of RyR2 causing a gain in SR Ca^{2+}-load and SR Ca^{2+}-cycling [10]. S100A1 has recently been shown to enhance SR Ca^{2+} signalling and contractile function of cardiomyocytes [11,12]. Additionally, S100A1 gene addition was shown to augment the isometric force generation of *in vitro* engineered heart tissue without interfering with basic regulatory mechanisms of muscular contractility [13]. The *in vivo* consequences of S100A1 overexpression in the heart has been demonstrated in transgenic mice and similarly to *in vitro* studies, cardiac-targeted S100A1 overexpression leads to enhanced left ventricular contractility and increased intracellular Ca^{2+} transients [14]. Interestingly, chronic S100A1 overexpression in transgenic mice was not accompanied by any detrimental effects on cardiac morphology or function [14].

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Gene transfer *in vivo* to the heart has significant therapeutic potential for several cardiovascular disorders. The delivery of a transgene encoding for S100A1 to the myocardium might be an attractive strategy for improving cardiac function in HF by replacing lost endogenous S100A1, thus increasing SR Ca^{2+}-cycling. However, since there are enormous differences between the transgenic mouse paradigm and exogenous gene transfer to adult animals, it is unknown whether the S100A1 gene delivery approach to larger animals would be effective to alter *in vivo* cardiac function. In this study, we sought to develop an intracoronary artery delivery method to accomplish myocardial S100A1 overexpression. In addition, we wanted to test the hypothesis that acute exogenous S100A1 gene transfer can alter *in vivo* cardiac function.

Methods

Adenoviral Constructs

The construction, production, and purification of bicistronic adenoviral constructs with a first-generation E1/E3-deleted, replication deficient adenovirus type V have previously been described [15]. The used adenoviral construct contained both the human S100A1 cDNA and the green fluorescent protein (GFP) cDNA as a reporter gene each under the control of a separate cytomegalovirus (CMV) promotor (AdvS100A1). A virus containing only the GFP cDNA cassette served as a control construct (AdvGFP).

In vivo Intracoronary Delivery of Adenoviruses

The Animal Care and Use Committee of Duke University Medical Center and of the Medical University of Luebeck approved all procedures performed in accordance with the regulations adopted by the National Institutes of Health. Gene delivery was performed in adult male New Zealand white rabbits (~2.5 kg) as described previously [16]. Briefly, rabbits were anesthetized with a mixture of ketamine (50mg/kg) and xylazine (5mg/kg), intubated and mechanically ventilated. After a right thoracotomy the pericardium was dissected and the aortic root exposed. Next, adenosine (0.75 mg/kg) was delivered to slow the heart. The adenoviral constructs (5×10^{11} total viral particles (tvp) of either AdvS100A1 or AdvGFP in 1.5 ml saline) or saline alone were rapidly injected into the left ventricular chamber while simultaneously clamping the ascending aorta. After 45 seconds the circulation was reestablished and closure of the thoracotomy was performed.

Western Blotting

Detection of S100A1 protein expression was performed as previously described using a custom-made antibody raised in rabbit against human S100A1 [17]. Optical density (OD*cm) of scanned chemiluminescence blots were evaluated using 1D-Scan and means were calculated from 3 representative blots. Values are expressed as percent of AdvGFP-treated controls.

Histology

Hearts were excised, rinsed in PBS, frozen in embedding compound, and stored at -80°C. Specimens were mounted on a freezing microtome, and 20 µm sections were transferred to glass slides. Transfection efficiency of *in vivo* gene transfer was assessed by GFP fluorescence in LV cryosectioned tissue. To confirm specifity of the GFP emission, the same offset for suppressed background of non-transfected hearts excited at 488 nm was applied to AdS100A1 and AdGFP treated hearts.

In vivo Hemodynamic Measurements

To assess contractile function, rabbits were sedated with a mixture of ketamine (50 mg/kg) and xylazine (5mg/kg) and a 2.5 french micromanometer (Millar Instruments Inc, Houston, USA) was introduced into the left ventricular (LV) chamber as described [16]. LV pressure data were determined under basal conditions and after infusion of 0.1, 0.5 and 1.0 µg isoproterenol kg/BW/min using a PC-assisted system (Bemon-Tip program, Hanau, Germany). All hemodynamic data reported correspond to a one minute average of steady state cardiac cycles.

Statistical Analyses

All data are expressed as mean ± SEM. Protein expression data was analysed via a student's t-test and *in vivo* hemodynamic data were compared by ANOVA. For all analyses, a value of $P<0.05$ was considered to be statistically significant.

Results

In vivo Myocardial Transgene Delivery

Intracoronary delivery of 5×10^{11} tvp of both AdS100A1 and AdGFP resulted in global and patchy transfection of the myocardium as described previously [16]. Figure 1 shows representative expression of the co-transfection marker GFP in LV myocardium 7 days following AdvS100A1 gene delivery.

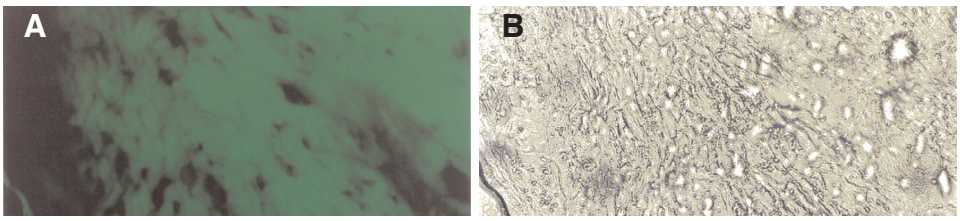

Fig. 1. Intracoronary S100A1 *in vivo* gene transfer. Representative GFP fluorescence microscopy (A) and corresponding light microscopy (B) of LV myocardium 7 days *in vivo* gene transfer. magnification, 10 x

Western Blot Analysis

Figure 2A and 2B illustrate representative western blot analysis carried out for the human isoform of S100A1 from left ventricular homogenates. When assessing S100A1 protein expression after AdvS100A1 delivery by western blotting of LV homogenates, we found a significant 3.2 ± 0.6 fold increase in S100A1 levels compared to the endogenous levels (Fig. 2). Endogenous S100A1 protein levels in the LV did not differ between AdvGFP and saline treated (Sham) control groups (Fig. 2A). Expression levels of Calsequestrin were found to be unchanged in all groups (data not shown).

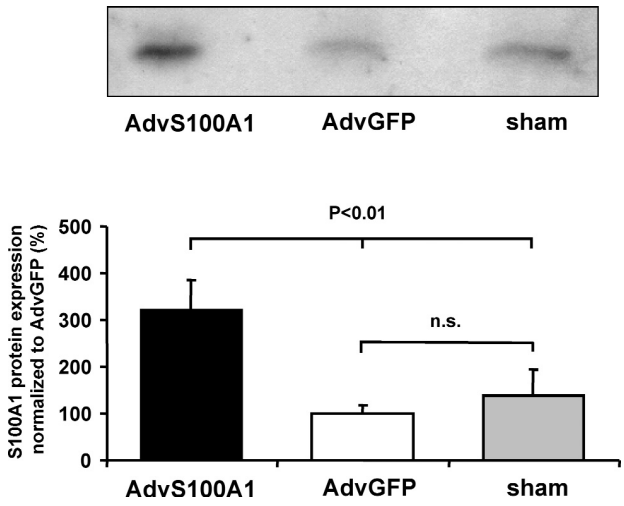

Fig. 2. Representative Western Blot of S100A1 protein levels in LV homogenates. Histogram of mean ± SEM (n = 3 each) LV S100A1 protein levels 7 days after AdvS100A1 delivery compared to control groups (AdvGFP and saline-treated).

Effects of S100A1 Overexpression on Myocardial Function

The goal of this study was to test whether *in vivo* myocardial S100A1 gene delivery could alter global heart function. To do this, we evaluated in vivo hemodynamic parameters of cardiac function by assessing values for left ventricular (LV) $+dP/dt_{max}$ and LV $-dP/dt_{min}$ as measurements of cardiac contractility and relaxation as well as LV systolic ejection pressure (SEP) and heart rate (HR). S100A1 overexpression as a result of the intracoronary delivery of AdvS100A1 leads to a significant increase of global contractile cardiac function. Animals subjected to injections of AdvGFP (n = 11) tended towards decreased contractile function compared to saline injected controls (n = 11), however, the differences did not reach the level of statistical significance (data not shown). Under basal conditions, hemodynamic parameters of AdvS100A1 treated rabbits (n = 8) did not differ significantly compared to both control groups (Fig. 3A-D). In contrast, after administration of 0.1μg/kgBW/min of isoproterenol, HR was not significantly increased in AdvS100A1-treated rabbits; however $+LVdP/dt_{max}$, $-LV\ dP/dt_{min}$, and LV SEP were significantly enhanced in S100A1 overexpressing rabbits compared to the AdvGFP treated control group ($+LVdP/dt_{max}$: 4756.6 ± 178.9 mmHg/sec vs. 3773 ±

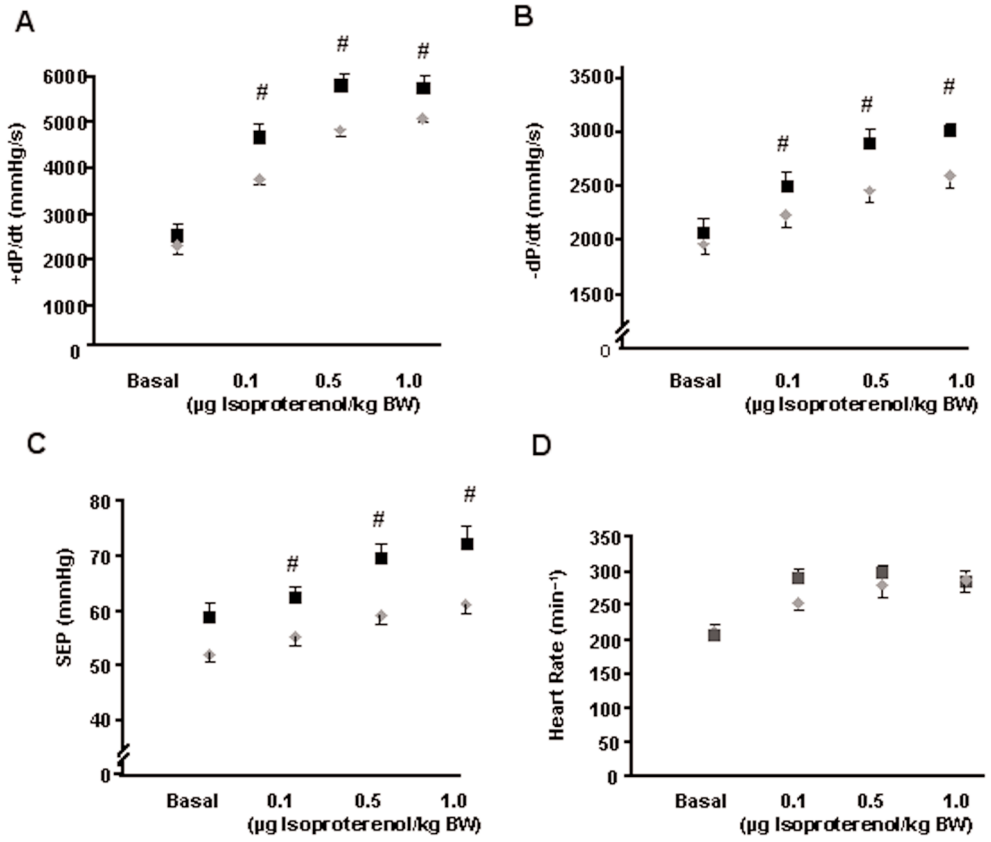

Fig. 3. S100A1 overexpression increased *in vivo* cardiac function: Cardiac function was assessed 7 days after S100A1 gene delivery. (A) +LV dp/dt max ; (B) -LV dp/dt_{min} and (C) systolic ejection pressure (SEP). (D) Heart rate remained unchanged. Shown are the mean ± SEM for AdvS100A1 (black rectangle; n = 8) and AdvGFP (grey diamond; n = 11). # AdvS100A1 vs. AdvGFP; P<0.05.

185.4 mmHg/ sec; P<0.005, –LV dP/dt_{min}: 2564 ± 111.1 mmHg/sec vs. 2237 ± 98.9 mmHg/sec; P<0.05, LV SEP: 65.2 ± 3.8 mmHg vs. 55.2 ± 1.8 mmHg; P<0.05, HR: 279.6 ± 10 min^{-1} vs. 249 ± 7.6 min^{-1}; ns.) (Fig. 3A-D). Importantly, enhanced hemodynamic parameters of AdvS100A1 treated rabbits remained significantly increased at higher and maximal b-adrenergic stimulation (0.5μg and 1.0 μg isoproterenol/ kgBW/ min) compared to AdvGFP treated controls (Fig. 3A-D).

Discussion

S100A1 has been characterized as a novel positive inotropic factor and regulator of myocardial contractility *in vitro* and *in vivo* [11-14]. Effects mediated by the S100A1 protein were mainly due to an increased SERCA2a activity and an augmented open probability of the RyR causing an overall gain in SR Ca^{2+} cycling [10, 14, 18-21]. Adenoviral-mediated overexpression of S100A1 was shown to augment isometric force generation of *in vitro* engineered heart tissue without interfering with basic regulatory mechanisms of muscular contractility [13]. Importantly, chronic cardiac inotropic effects observed in S100A1 transgenic mice were not accompanied by any signs of hypertrophy, fibrosis or other pathology [14]. Overall, results with cardiac S100A1 targeted expression suggest that S100A1 overexpression might be supportive to failing myocardium and enhance global LV function. Of added importance to the role of S100A1 in heart function, data generated from S100A1 knockout mice have shown that normal S100A1 protein levels are required for the cardiac reserve to adapt to acute and chronic hemodynamic stress *in vivo* [22].

In this study, we demonstrate that acute global myocardial overexpression of the human S100A1 protein can lead to an enhancement of cardiac *in vivo* physiology after intracoronary administration of recombinant adenovirus. Given the significant down-regulation of S100A1 protein in end-stage human HF [8], our present data provide a basis that gene transfer to replace lost S100A1 in end-stage HF may have potential to support the failing heart. Noteworthy, S100A1 overexpressing rabbits exhibit an increased contractile reserve while basic cardiac function remained unchanged. This result contrasts our *in vitro* findings as well as our findings in S100A1 transgenic mice [11-14]. This might be explained by β-adrenergic receptor independent effects on contractility with increased S100A1 expression and a lower and non-homogenous S100A1 overexpression in myocardium. Moreover, the current *in vivo* study was done in healthy rabbits without prior cardiac dysfunction and S100A1 overexpression may have a more profound impact on cardiac contractility in the failing heart, when the endogenous S100A1 is down-regulated. In addition, SR dependent Ca^{2+}-cycling differentially contributes to myocardial function in rodents and rabbits and this might explain, at least in part, different findings for basal cardiac function in S100A1 overexpressing mouse and rabbit models.

This study demonstrates that S100A1 overexpression in the context of healthy myocardium can lead to increases in cardiac contractility, however, our ultimate goal was to employ the strategy of restoration of cardiac S100A1 expression levels in the failing heart. Therefore, in a continuative study, the impact of an adenoviral S100A1 gene therapy approach was tested in a rat heart failure model *in vivo* [10]. Twelve weeks after cryoinfarct, rats with heart failure were treated with either the AdvS100A1 or the AdvGFP vector using the global intracoronary delivery method. Rats were studied 7 days after gene transfer. Western blot data from these hearts show that down-regulated S100A1 that is present in these failing hearts is completely normalized 7 days after AdS1001 gene delivery [10]. LV hemodynamics including HR, $+dP/dt_{max}$ and $-dP/dt_{min}$ as measures of LV contractility and relaxation as well as LV end-diastolic pressure (LVEDP) and LV systolic pressure (LVSP), were studied. The depressed contractility observed in this HF model was reversed one week after S100A1 normalization subsequent to AdS100A1 gene delivery to the myocardium [10]. Mechanistically at the myocyte level, this was due to an increased SERCA2a activity and a biphasic effect on the open probability of RyR2 causing a gain in SR Ca^{2+}-load and SR Ca^{2+}-cycling [10].

However, one limitation of this study is the short observation period of only one week after gene transfer which is a consequence of the transient gene expression due to the use of a first generation adenovirus. Further studies using advanced adenoviral vectors are now needed to investigate, if exogenous myocardial S100A1 gene delivery will permanently support LV function. Moreover, to clinically implement S100A1 gene therapy as a potential therapeutic modality, potential immunogenicity associated with adenoviral gene delivery system has to be addressed very carefully [23]. Recently developed adeno-associated viral systems are not expected to cause an immune response and inflammation and this feature might increase the duration of therapeutic gene expression *in vivo* [24, 25].

The study provides two novel findings. The first is that S100A1 *in vivo* gene delivery to the myocardium is feasible. Second, we showed that S100A1 gene transfer to the myocardium of larger animals using the intracoronary gene delivery approach can lead to an increased cardiac contractility. This is especially important since there are enormous differences between the transgenic mouse paradigm and the exogenous gene transfer to adult animals. Since Ca^{2+}-cycling in rabbit myocardium apparently reflects relative SR dependence in human hearts, this study has important impact for future clinical S100A1 gene therapy trials. Importantly, this report provides a basis for a continuative study in which failing rats underwent S100A1 gene therapy. In this study we could show that normalization subsequent to AdS100A1 gene therapy to the myocardium rescued heart failure in rats. In conclusion, S100A1 might be a therapeutical target and complement the established treatment in HF.

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