ULTRASOUND TARGETED MICROBUBBLE DESTRUCTION AUGMENTS AAV-MEDIATED CARDIAC GENE TRANSFER IN RATS

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1 INTRODUCTION

Cardiac gene transfer has been considered as a promising therapeutic tool in cardiology. While our knowledge in molecular mechanisms of cardiac diseases has grown exponentially, development of efficient and feasible gene transfer has not been able to match that pace. Most delivery systems have been based upon invasive procedures, such as direct intramyocardial injection and prolonged or enhanced coronary perfusion. However, these techniques have inherent risks that may outweigh their benefit. Therefore, the development of non-invasive but highly organ specific systemic gene transfer approaches has been a major focus. Presently, one of the most promising vector systems for non-invasive cardiac gene transfer is the adeno-associated virus (AAV)[1,2]. However, the use of pseudotyped and transcriptionally targeted AAV vectors for systemic application in larger animals is still limited by the high amount of vector necessary for efficient transduction and gene expression, even if cell-type specific promoters are used. Therefore, a mechanical but non-invasive targeting of AAV vectors would be advantageous. These aspects could be combined by a technique that has evolved as a new site specific targeting tool in gene and drug delivery: ultrasound targeted microbubble destruction (UTMD). It is based upon gas-filled microbubbles that are used as ultrasound contrast agents. When sonified with ultrasound in their resonance frequency (between 1 and 2 MHz), high mechanical index ultrasound will induce high amplitude oscillations that lead to microbubble destruction. While this phenomenon can be used to characterise refill kinetics of tissue perfusion, it can also be used to deliver a microbubble-loaded bioactive substance into an organ[3-5]. In addition, UTMD benefits from secondary effects of microbubble destruction that will increase capillary permeability in their near vicinity[6]. Based on these phenomena, both adenoviral vectors and plasmid DNA were successfully delivered to the heart.
Therefore, the aim of this study was to investigate whether UTMD could augment AAV mediated gene transfer to the heart.

2 METHODS

2.1 Microbubbles

AAV-loaded microbubbles were produced by a modification of a previously described method [23]. One half ml of a solution containing $4x10^{10}$ genomic particles AAV-6 or AAV-9, 1% DL-α-phosphatidylcholine (Sigma, Munich, Germany), 0.25% DL-α-phosphtidylethanolamine (Sigma, Munich, Germany) and 10% glycerol in PBS was incubated at 20°C for 10min. It was then transferred to a tube filled with octafluorpropane gas and shaken for 20 sec at 4300 oscillations per minute (Capmix, 3M, St. Paul, MN, USA). Microbubble size and concentration were measured prior to injection (Multisizer 3, Beckman-Coulter, Fl, USA).

2.2 In vivo gene transfer

Rats were anesthetized with an intra peritoneal injection of 400mg/kg 2,2,2-tribromethanol (Sigma, Munich, Germany). A polyethylene tube was inserted into the right internal jugular vein. After shaving and depilating the chest, the transducer was placed on the thorax in a mid short axis view with a 1.5 cm gel stand-off. The microbubble suspension (in one ml) or pure virus suspension was infused at a constant rate of 3ml/h for 20 minutes. Microbubble destruction was obtained using a Sonos 5500 machine (Philips, Eindhoven, Netherlands) with an S3 transducer operating in ultraharmonic mode (transmit 1.3 MHz) at a mechanical index of 1.6. Sonography was ECG-triggered, at 80 ms after the peak of the R-wave, to deliver a burst of 4 frames of ultrasound every 4 cardiac cycles. At the end of the experiment the jugular vein was tied off and the skin closed. After four weeks animals were euthanized and various organs were harvested and either frozen in liquid nitrogen for luciferase assays or fixed for histologic analysis.
Various protocols were applied to test the effect of UTMD on cardiac transduction. Using luciferase encoding AAV-6, six rats received microbubbles loaded with $4x10^{10}$ genomic particles and ultrasound to the heart, and six rats received $4x10^{10}$ genomic particles in PBS (without microbubbles) and ultrasound to the heart. Using luciferase encoding AAV-9, four rats received microbubbles loaded with $4x10^{10}$ genomic particles and ultrasound to the heart, and four rats received $4x10^{10}$ genomic particles in PBS (without microbubbles) and ultrasound to the heart.
Using GFP encoding AAV-9 four rats received microbubbles loaded with $1x10^{11}$ genomic particles and four rats received $1x10^{11}$ genomic particles in PBS and ultrasound to the heart.

3 RESULTS AND CONCLUSION

In AAV-6 treated rats, left ventricles demonstrated a significantly higher transgene expression (6-fold) in the UTMD group, compared with the control group receiving AAV without microbubbles in the presence of ultrasound (151705±76783 vs. 24563±24751, $p<0.05$) (Figure 1A). For the right ventricle, UTMD resulted in a trend towards higher reporter activities (5-fold; 101903±78558 vs. 18805±24135, $p=0.21$) with a similar result in atria (3-fold; 4681±3897 vs. 1406±3146, $p=0.18$). Expression in other organs was close to background except for the liver which revealed significant luciferase activities in both groups most probably due to high tropism of AAV-6 vectors for rat liver.
As with AAV-6, AAV-9 pseudotyped vectors loaded on microbubbles resulted in significantly higher transduction levels in left ventricular myocardium (23-fold) compared to AAV-9 without microbubbles (326427±198012 vs. 13900±15094, $p<0.05$) (Figure 1B). Although not significant, there was a trend towards higher reporter gene activities in right ventricle (7-fold; 52476±42194 vs. 7683±4570, $p=0.12$), atrium (2-fold; 18328±21351 vs. 9070±13281, $p=0.49$) and liver (4-fold; 49742±33213 vs. 11543±16487, $p=0.10$) with the AAV-9/microbubble mixture compared to controls.
In order to characterize spatial distribution of gene transfer with ultrasound targeted microbubble destruction, we analyzed rats that were subjected to gene delivery with AAV-9 vectors encoding an EGFP reporter gene either with or without microbubbles in the presence of ultrasound. While only few green fluorescent cells could be detected in control animals (having received virus and ultrasound without microbubbles), EGFP positive cells could be detected all over the myocardium, especially in the anterior wall of the left ventricle after UTMD treatment.
In conclusion, our study shows for the first time the potential of UTMD to target AAV-vectors into rat hearts. Of note, this is a non invasive technique that can be applied by intravenous, systemic administration of the vector, in contrast to direct intramyocardial or intracoronary injection. Using the combination of AAV loaded microbubbles and ultrasound, it is possible to downscale the amount of virus. Thus, it may offer the possibility to use systemic administration of AAV vectors even in humans, without the necessity to use unrealistically high doses of viral particles. However, further work is essential to transfer this approach to a larger animal model and to use functional/therapeutic genes that would cause a physiologic response.

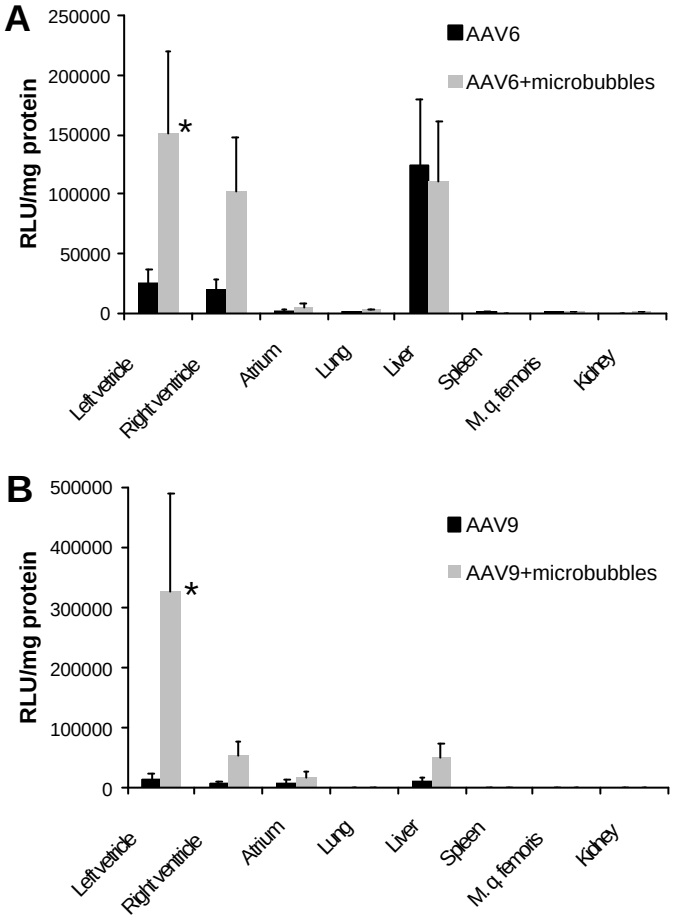

Figure 1: Effect of ultrasound targeted destruction of microbubbles loaded with AAV-6 (A) and AAV-9 vectors (B) on transduction efficiencies of representative organs in adult rats. *: $p<0.05$ AAV-microbubbles vs. AAV.

4 REFERENCES

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