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The use of cationic microbubbles to improve ultrasound-targeted gene delivery to the ischemic myocardium

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ABSTRACT

We synthesized a cationic microbubble (CMB) with the aim of enhancing its DNA-carrying capacity to improve targeted gene transfection of the ischemic heart for cardiac regeneration. We previously reported that ultrasound-targeted microbubble destruction (UTMD) employing the commercial Definity microbubble (MB) successfully transfected genes into rodent hearts, but the transfection efficiency was modest. We synthesized a CMB and compared its DNA-carrying capacity and reporter gene transfection efficiency with the Definity MB. The CMB bound 70% more plasmid DNA than the Definity MB. UTMD-mediated gene delivery with the CMB enhanced both transfection efficiency and gene expression. In vivo studies assessed the ability of the CMB to deliver the therapeutic *AKT* gene to the ischemic rat myocardium and evaluated the effects on apoptosis, angiogenesis, and cardiac function. *AKT* transfection with the CMB reduced infarct size ($p < 0.05$), increased infarct thickness ($p < 0.05$), reduced apoptosis ($p < 0.05$), increased vascular density ($p < 0.05$), and improved cardiac perfusion and function ($p < 0.05$) compared to the Definity MB. Delivery of *AKT* with the CMB resulted in greater cardiac functional improvements compared to the Definity MB. UTMD therapy with this CMB provides an efficient platform for the targeted delivery of factors required to regenerate the ischemic heart and preserve cardiac function.

1. Introduction

Following a myocardial infarction (MI), cardiomyocyte loss can be associated with a cascade of interrelated events that culminate in ventricular decompensation and heart failure. Despite advances in clinical management, ventricular dysfunction and heart failure remain the major causes of morbidity and mortality following an MI [1,2]. Gene therapies have been demonstrated to enhance angiogenesis and cell survival as well as reduce adverse cardiac remodeling and improve recovery of ventricular function [3,4]. However, inefficient gene delivery to the target tissue has limited the potential of this therapy. Ultrasound-targeted microbubble destruction (UTMD) can provide minimally invasive, repeatable, and targeted gene delivery to the infarcted myocardium to restore ventricular function.

Intravenously administered lipid microbubbles (MBs) are currently utilized for the clinical evaluation of myocardial perfusion. During the past decade, the UTMD technique has been used to deliver genes to the hearts of animals [5–7]. Recently, this technique was also used to target delivery of a growth factor to the ischemic rat myocardium [8]. We previously demonstrated that UTMD-mediated gene therapy improved myocardial perfusion and cardiac function following MI in mice [6] and rats [7]. We found that UTMD gene delivery is a controlled DNA delivery technique that is less invasive and more effective at targeted delivery than other currently available approaches. The main limitation of UTMD-mediated gene transfection in previous studies was the modest transfection efficiency of the commercially available, weakly cationic Definity MB, which has a low DNA-binding capacity [9]. Therefore, we synthesized a positively charged MB with a greater

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capacity to bind DNA and hence a predicted improvement in UTMD-mediated gene transfection efficiency.

An appealing candidate for cardiac gene therapy is the protein kinase AKT, which is activated by hypoxia and regulates cell survival and angiogenesis [10]. Overexpressing the *AKT* gene promotes cardiac cell survival, enhances paracrine effects, and stimulates neovascularization around the infarct by upregulating survivin, which is an inhibitor of apoptosis, and by downregulating the pro-apoptotic BCL2-associated death promoter (BAD) protein. AKT phosphorylates BAD, causing it to dissociate from the BCL2/Bcl-X complex and lose its pro-apoptotic function [11–13]. Cells overexpressing *AKT* improved cardiac function when implanted into the injured heart [14]. Global overexpression of *AKT* in injured myocardium may enhance cardiac repair and tissue regeneration.

In this study, we describe the synthesis of a cationic microbubble (CMB) to increase the DNA-binding capacity compared with the commercially available Definity MB. We hypothesize that this CMB will enhance gene transfection efficiency. We utilized this CMB to deliver the *AKT* gene to the ischemic rat myocardium via UTMD and assessed AKT protein expression and the effects on angiogenesis, apoptosis, and ventricular function.

2. Materials and methods

2.1. Preparation of the CMB

The CMB was prepared by the method of thin film hydration [15]. Hydrogenated soy L-α-phosphatidylcholine (HSPC) and 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA) (Avanti Polar Lipids Inc., Alabaster, AL, USA) were weighed and dissolved in a flask with chloroform at a molar ratio of 94:6. The flask was placed on a rotary evaporator that was vacuumized to remove the organic solvent, forming a thin lipid film. The mixture was incubated under vacuum and dry conditions overnight. Phosphate-buffered saline (PBS) was added and the mixture was hydrated on the rotary evaporator. The mixture was sonicated with octafluoropropane (C_3F_8) gas (Airgas Inc., Calgary, AB, Canada) to prepare the CMB suspension. The C_3F_8 gas was bubbled through the solution for 1 min before sonication. Sonication was performed at 20 kHz, 33–42 W, with a Sonicator 3000 (Misonix Inc., Farmingdale, NY, USA). The CMBs were stable in suspension for up to one month at 4 °C.

2.2. Characterization of the MBs

DEFINITY® MBs were purchased from Bristol-Myers Squibb Canada (Montreal, QC, Canada). The zeta potential ($n = 10$ batches/group) of the MBs was measured by dynamic light scattering using a Zetasizer Nano ZS90 (Malvern, Worcestershire, UK). The size of the MBs ($n = 5$ batches/group) was determined by electrozone sensing using a Multisizer 4 Coulter Counter (Beckman Coulter, Mississauga, ON, Canada). The morphology was examined under light microscopy.

2.3. Evaluation of MB DNA-binding capacity

For this assay, 10, 20, 40, or 80 μg of luciferase plasmid DNA (2 μg/uL) in 5, 10, 20, or 40 μL of TE buffer was diluted with saline to a total volume of 50 μL and was added to 50 μL of MB suspension (100 μL in total) and incubated for 10 min. To obtain the accurate quantity of bound DNA, the microbubble fraction was collected and the plasmid DNA on the microbubbles was separated and precipitated for analysis. The samples were centrifuged at 1000 × *g* to form two phases: an upper, milky white layer containing the MBs and a lower, clear layer. The two phases were separated, and the plasmid DNA was precipitated with 3 M sodium acetate and 100% ethanol. After another centrifugation, the DNA pellet was washed with 70% ethanol and re-suspended with TE buffer to measure the DNA concentration in each layer using optical density. The percentage of plasmid DNA in the MB layer was calculated using the following formula: $\% = (DNA_{MB}/\text{total DNA}) \times 100$.

2.4. Plasmid DNA and MB solutions

We used pcDNA3 plasmids containing either the firefly luciferase gene or human *AKT* gene under the control of a cytomegalovirus promoter (or empty pcDNA3 plasmid for the vector control). For in vivo studies, the amount of plasmid DNA was based on body weight (0.20 mg/kg body weight). Therefore, 40–45 μg of plasmid DNA (0.5 μg/uL) in 80–90 μL of TE buffer was diluted with saline to a volume of 100 μL and then mixed with an equal volume of microbubbles (200 μL in total) for 20 min at room temperature. The MB solutions were diluted with saline to a total volume of 0.4 mL/rat.

2.5. Animal model

Sprague–Dawley rats (weighing 200–225 g) were obtained from Charles River Laboratories (Saint-Constant, QC, Canada), and ischemia/reperfusion (I/R) was generated under general anesthesia by occluding the left anterior coronary artery for 60 min followed by reperfusion, as previously described [7]. The Animal Care Committee of the University Health Network approved all animal procedures. Experiments were performed according to the *Guide to the Care and Use of Experimental Animals* from the Canadian Council on Animal Care.

2.6. Experimental design and timeline

For the reporter gene study, the luciferase plasmid was delivered to the heart by UTMD 3 days after I/R, and luciferase expression and activity within the myocardium were measured over 14 days.

For the *AKT* gene study, echocardiography was performed 5 days after I/R to measure infarct size. The experimental timeline is available in Supplemental Fig. 1. To obtain rats with a similar infarct size, those with an akinetic left ventricular wall length <0.8 cm or >1.2 cm were excluded from the study. The remaining rats were randomly assigned to the vector control, CMB, or Definity MB group. Control animals received empty plasmid via UTMD with the Definity MB, and treated animals received the *AKT* plasmid via UTMD with either the CMB or Definity MB 5 days after I/R. Hearts were collected for TUNEL and Western blot analysis 3 days after UTMD-mediated plasmid delivery. For histology and immunostaining, hearts were collected 16 days after UTMD-mediated plasmid delivery (21 days after I/R).

2.7. UTMD delivery

Rats were sedated with 2% isoflurane, and the plasmid–MB solution was infused into the tail vein at a rate of 1.2 mL/h. Simultaneously, an ultrasound beam was delivered with an M3S transducer using a Vivid 7 system (GE Healthcare, Milwaukee, WI) operating in the second harmonic mode (transmit: 1.6 MHz; receive: 3.2 MHz) with an electrocardiograph (ECG) trigger at every fourth end-systole for 20 min. The depth was set at 3 cm, and the transducer was adjusted with a gel interface so that the focus was positioned at the myocardial level. A mechanical index of 1.3 was employed. Each ultrasound burst was seen to eliminate a large number of the MBs in the myocardium, and a pulsing interval of four cardiac cycles allowed replenishment of the MBs before the next ultrasound burst.

2.8. Bioluminescence imaging and luciferase activity

Luciferase expression (photons/s/cm^2/steradian) in live rats was monitored by bioluminescence imaging using the Xenogen IVIS Spectra System and the Living Image Program (Xenogen, Hopkinton, MA, USA) at 3, 7, and 14 days after UTMD. Rats were anesthetized with 2.5% isoflurane, and 5 min later, 150 mg/kg body weight of D-luciferin (Xenogen) was injected intraperitoneally. Rats were imaged 5 min later according to the manufacturer's instructions.

Luciferase activity was assayed at 3 and 14 days after UTMD. The animals were euthanized, and the hearts were perfused with normal saline, removed, snap-frozen in liquid nitrogen, and stored at −80 °C. Luciferase activity assays (BD Biosciences, Mississauga, ON, Canada) were performed according to the manufacturer's instructions. Luminescence was measured over 10 s. Luciferase activity was expressed as relative light units (RLU)/min/mg heart mass.

2.9. Myocardial function, perfusion, and infarct measurement

Myocardial perfusion was evaluated using myocardial contrast echocardiography on days 5 and 21 after I/R. Rats were sedated, and the MB solution was injected through the tail vein. Digital images were used to measure signal intensity offline at a pulsing interval of 1800–2000 ms (plateau intensity). The anterior wall represented the infarct region, and the posterior wall represented the non-infarct (normal) region. The intensity ratio was calculated (signal intensity in the anterior wall divided by that in the posterior wall) to estimate myocardial blood volume.

Using echocardiography, left ventricular end-diastolic and end-systolic diameters and volumes were evaluated on the day of I/R (prior to the procedure), and then 5 and 21 days after I/R. Infarct length and thickness were measured on day 21 after I/R. Infarct length was expressed as a percentage of the left ventricular circumference, measured on the short-axis view of the left ventricle at the mid-papillary muscle level during end-diastole. Infarct thickness (in mm) was measured in the mid-portion of the infarct.

2.10. Myocardial AKT, phospho-AKT, survivin, and phospho-BAD expression

Hearts were snap-frozen on day 3 after UTMD-mediated plasmid delivery. The heart homogenate was treated with an appropriate extraction buffer, and protein expression of AKT, phospho-AKT (Ser473), survivin, and phospho-BAD was measured by Western blot [16]. Each sample contained tissue from the infarct and border zone regions.