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The use of MMP2 antibody-conjugated cationic microbubble to target the ischemic myocardium, enhance Timp3 gene transfection and improve cardiac function

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ABSTRACT

The objective of this study was to synthesize a cationic microbubble (CMB) conjugated with an antibody against matrix metalloproteinase 2 (CMB_{MMP2}) to increase microbubble accumulation and gene transfection in the infarcted myocardium and to restore ventricular function following an ischemic insult. We previously reported that our CMBs enhanced the efficiency of gene transfection following ultrasound-targeted microbubble destruction (UTMD) in rodent hearts. Therefore, we conjugated a thiolated MMP2 antibody to the PEG chains on the CMB surface, which was verified by fluorescent microscopy. Rats underwent ischemia/reperfusion injury 3 days prior to UTMD delivery of the control or *Timp3* plasmid. The CMB_{MMP2} improved microbubble accumulation in the infarct region, with 57% more contrast intensity compared to the non-conjugated CMB. UTMD-mediated CMB_{MMP2} delivery of the *Timp3* gene significantly increased TIMP3 protein levels in the infarct scar and border zone at 3 days post-UTMD compared to delivery by the non-conjugated CMB. Both MMP2 and MMP9 activity were reduced in the CMB_{MMP2} *Timp3* group, which resulted in smaller and thicker infarcts and improved cardiac function. UTMD therapy with this CMB_{MMP2} provides an efficient platform for the targeted delivery of factors intended to preserve ventricular structure and improve cardiac function after ischemic injury.

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1. Introduction

Ischemic cardiac injury is the main reason for heart failure. Despite the advances in clinical management, ventricular dysfunction and heart failure remain the major causes of morbidity and mortality following ischemic injury [1,2]. Increased cardiac matrix metalloproteinase (MMP) activity has been reported in both patients with end-stage heart failure [3,4] and in experimental models of heart failure [5]. Inhibition of MMP activity by tissue inhibitors of matrix metalloproteinases (TIMPs) has been shown to reduce ventricular remodeling and improve cardiac function following ischemic injury [4,6,7].

Gene therapy has been explored to treat a variety of conditions, including myocardial infarction (MI) and heart failure [8,9]. Microbubbles, which were designed for molecular imaging and are used as ultrasound contrast agents for visualizing perfusion, have recently been adapted as a vehicle for delivering genes, proteins, or drugs because of their visibility in the target tissue [10,11]. Microbubble destruction provides a more targeted technique for gene delivery than traditional intravenous injection.

Commercially available microbubbles are close to neutral in charge and have a limited capacity to carry sufficient plasmid to achieve a therapeutic effect. Modification of microbubbles to create a positively charged surface has been shown to enhance gene delivery by ultrasound-targeted microbubble destruction (UTMD) [12–14]. These cationic microbubbles (CMBs) protect the bound DNA against nucleases [14,15]. We previously synthesized a CMB and reported that UTMD employing our CMB enhanced gene transfection efficiency in rodent hearts compared with the commercially available Definity microbubble [13]. Another

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approach to increase the therapeutic potential of microbubbles is to specifically target them to the tissue of interest by conjugating receptor ligands or antibodies to their surface. For example, microbubbles conjugated with a luteinizing hormone–releasing hormone analog have been used to target human ovarian cancer cells that express receptors for the hormone [16], and microbubbles with an ICAM-1 antibody on their surface can specifically bind to activated endothelial cells overexpressing this protein [17].

To enhance tissue targeting in the current study, we conjugated an antibody against MMP2 onto our CMB to increase the accumulation of the microbubbles in the infarcted myocardium of rats. Following myocardial ischemic injury, the ischemic region has increased levels of MMPs, which permits specific targeting of microbubbles to the tissues expressing these proteins. Hence, we hypothesized that this MMP2 antibody-conjugated CMB (CMB_{MMP2}) would improve UTMD-mediated gene transfection in the ischemic heart.

An appealing candidate for cardiac gene therapy is *Timp3*, which is a strong inhibitor of MMP2 and MMP9. Increased MMP levels after MI participate in the remodeling of the extracellular matrix [3,4]—a dynamic scaffold that supports cardiomyocytes and facilitates contraction. Matrix remodeling promotes ventricular dilation and dysfunction [18]. Re-establishing the MMP/TIMP balance by increasing TIMP expression has been shown to mitigate matrix remodeling and treat ischemic cardiomyopathy following an MI [19]. Cells overexpressing *Timp3* have been reported to improve cardiac function when implanted into the injured heart of rodents [7,20]. In this study, we describe the synthesis of the CMB_{MMP2} to specifically target delivery of the matrix-modulating *Timp3* gene to the infarct region of the rat heart. We hypothesized that UTMD using this CMB_{MMP2} would enhance gene delivery and expression compared with the non-conjugated CMB, to improve cardiac repair and ventricular function after an MI.

2. Materials and methods

2.1. Microbubble preparation

The CMBs were formulated with hydrogenated soy L-α-phosphatidylcholine (HSPC), 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA), and distearoyl phosphatidylethanolamine-polyethylene glycol 2000-maleimide (DSPE-PEG2000-maleimide; Avanti Polar Lipids, Alabaster, AL) at a molar ratio of 92:6:2, as previously reported [21]. The CMB_{MMP2} was prepared via thiol-mediated bioconjugation [22]. Briefly, thiolation of the mouse MMP2 antibody (ProteinTech, Chicago, IL) was performed using a 10-fold molar excess of Traut's reagent (Thermo Scientific-Pierce, Rockford, IL) in PBE buffer (1 mM EDTA in PBS, pH 8.0) at room temperature on a bench-top rotator for 1 h. Excess Traut's reagent was removed with a Zeba Desalt Spin Column (Thermo Scientific-Pierce) equilibrated with phosphate buffer (pH 6.0). The degree of thiolation (>90%) was controlled with Ellman's reagent (Thermo Scientific-Pierce), according to the supplier's instructions.

Subsequently, the thiolated MMP2 antibody was reacted with the maleimide functional groups at the distal end of the PEG chains on the CMBs at a MMP2:maleimide molar ratio of 30:1 in an aqueous solution on a bench-top rotator at 4 °C for 24 h. To ensure that there were no unreacted maleimide groups, L-cysteine was added at a L-cysteine:maleimide molar ratio of 1000:1. Unconjugated MMP2 was removed by centrifuging the suspension at 10,000 rpm for 5 min. The conjugation of MMP2 antibodies to the CMBs was confirmed using the Bio-Rad Protein Assay, as previously reported [23].

2.2. Microbubble characterization

Following dilution in PBS, the size distribution and zeta potential of the microbubbles were measured by dynamic light scattering ($n = 10$ batches/group) using a Zetasizer 3000HS (Malvern, Worcestershire, UK).

To confirm conjugation of the MMP2 antibody to the CMBs, 100 μL of fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG (Abcam, Cambridge, MA) solution was added to 200 μL of aqueous $CMBs_{MMP2}$ (10 mg/mL) and thoroughly mixed for 30 min at 4 °C. The mixture was separated into two distinct layers: the lower layer was discarded, and the upper layer was washed three times with PBS to elute the free FITC-conjugated IgG. The microbubbles were observed under a fluorescence microscope (Axio Observer, Carl Zeiss, Jena, Germany).

2.3. Plasmid DNA and microbubble solutions

We used pcDNA3 plasmids (Life Technologies, Burlington, ON) containing either the firefly luciferase gene or mouse *Timp3* gene under the control of the cytomegalovirus promoter (or empty pcDNA3 plasmid for the vector control). For in vivo studies, plasmid DNA (0.20 mg/kg body weight) was incubated with the microbubbles (0.10 mL/rat) for 20 min at room temperature. The microbubble solutions were diluted with saline to a total volume of 0.4 mL/rat. The empty control plasmid was delivered via the non-conjugated CMB.

2.4. Animal model

Sprague–Dawley rats (weighing 200–225 g) were obtained from Charles River Laboratories (Saint-Constant, QC). Under general anesthesia, ischemia/reperfusion (I/R) was generated by occluding the left anterior coronary artery for 60 min followed by reperfusion, as previously described [13]. Experiments were performed according to the *Guide to the Care and Use of Experimental Animals* from the Canadian Council on Animal Care.

2.5. MMP2-targeted imaging in the ischemic heart

To assess the accumulation of microbubbles in the myocardium, increase the number of microbubbles in the cardiac interstitium, we performed MMP2-targeted imaging. However, in order to transiently increase microvascular permeability prior to microbubble injection and imaging, triggered myocardial contrast echocardiography (MCE) was first performed, as previously reported [24,25]. Three days post-I/R injury, the rats were sedated with 2% isoflurane, and 0.5 mL of the CMB solution was infused intravenously via the tail vein at a rate of 3 mL/h for 10 min. Triggered ultrasound was performed with the Vivid 7 ultrasound system (GE Healthcare, Milwaukee, WI) with a M3S transducer operating in the second harmonic mode (transmit: 1.6 MHz; receive: 3.2 MHz) with an electrocardiographic trigger at every fourth end-systole. This sequence produced the optimum microbubble signal in the myocardium on the imaging frame.

MMP2-targeted imaging was performed immediately after the above MCE procedure. Briefly, 0.2 mL of microbubble solution was slowly infused for 5 min to allow for interaction between the microbubbles and the MMP2 expressed in the heart tissue (CMB $n = 5$, CMB_{MMP2} $n = 6$). Ultrasound contrast images were taken 1 and 3 min after completion of the infusion. The intensity of the signals originating from the retained microbubbles was measured after 3 min, and the signal intensity in the infarct area was calculated as a percentage of that in the remote area, as previously reported [26,27].

2.6. UTMD-mediated gene delivery

Three days following I/R, rats were sedated with 2% isoflurane, and the plasmid–microbubble solution was infused into the tail vein at a rate of 1.2 mL/h for 20 min. Simultaneously, an ultrasound beam was delivered with an M3S transducer using the Vivid 7 system (GE Healthcare) operating in the second harmonic mode (transmit: 1.6 MHz; receive: 3.2 MHz) with an electrocardiographic 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 mid left ventricular level. A mechanical index of 1.3 was employed. Each ultrasound burst eliminated a large number of the microbubbles in the myocardium, and a pulsing interval of four cardiac cycles was employed to allow replenishment of the microbubbles before the next burst. We previously employed this method for UTMD gene delivery to the ischemic rat heart using our CMB [13].

2.7. Bioluminescence imaging and luciferase activity

Luciferase expression (photons/s/cm^2/steradian) was monitored in live rats by bioluminescence imaging using the Xenogen IVIS Spectra System and the Living Image Program (Xenogen, Hopkinton, MA) 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 ($n = 6$/group).

Luciferase activity was also assayed at 3, 7, 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) 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 (CMB $n = 4$, CMB_{MMP2} $n = 6$).

2.8. Myocardial function

Echocardiography was performed in M-mode in the short-axis view of the left ventricle to evaluate left ventricular internal end-diastolic and end-systolic diameters (LVIDd and LVIDs) on the day of I/R (prior to the procedure), and then 3 and 21 days after I/R ($n = 6$/group). Left ventricular end-diastolic volume (EDV) and end-systolic volume (ESV) were calculated using the following formulae: $EDV = 1.047 \times LVIDd^3$; $ESV = 1.047 \times LVIDs^3$. Ejection fraction was calculated as the following: $EF\ (\%) = (EDV - ESV)/EDV \times 100$.

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