The use of antibody modified liposomes loaded with AMO-1 to deliver oligonucleotides to ischemic myocardium for arrhythmia therapy

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Abstract

MicroRNA-1 (miR-1) has been found in cardiac and skeletal tissues. It is overexpressed in ischemic cardiac tissues. Down-regulation of miR-1 could relieve arrhythmogenesis by the anti-miR-1 antisense oligonucleotides (AMO-1). To increase the therapeutic efficiency and inhibit off-target effects of AMO-1, here we explored anti-cardiac troponin I (cTnI) antibody modified liposomes loading with AMO-1 (cT-A-LIP) to deliver the oligonucleotides to ischemic myocardium tissues. Liposomal cytotoxicity was assessed by MTT assay. The targeting abilities to foci were evaluated by \textit{in vivo} imaging. The uptake and bio-distribution \textit{in vitro} were observed by live cell station and flow cytometry, respectively. The anti-arrhythmic effects of cT-A-LIP \textit{in vivo} were evaluated by electrocardiograms (ECG), immunohistochemistry, real-time PCR and patch-clamp recording. Immunohistochemistry showed that cTnI expression had a peak at the third day after myocardial infarction (MI). After cT-LIP administration via tail vein, accumulation of fluorescent trackers in the ischemic foci was significantly increased more than that of LIP. In addition, after cT-A-LIP administration, the ischemic arrhythmias were recovered and ST segment in ECG was elevated nearly back to normal. Compared with MI group, miR-1 expression was significantly down-regulated while Kir2.1 and CX43 protein expression were increased. Patch-clamp recordings showed that cT-A-LIP as well as AMO-1 incubation increased K\textsuperscript{+} current density in guinea pigs ventricular cardiomyocytes acting on repolarized membrane potential. In conclusion, the cT-A-LIP not only delivered AMO-1 to ischemic myocardium in MI rats, but validated AMO-1 on relieving ischemic arrhythmia by silencing of miR-1 in ischemic myocardium and restoring the depolarized resting membrane potential (RMP) in MI rats.

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1. Introduction

Ischemic arrhythmia caused by myocardial infarction is one of the global public health problems with high morbidity and mortality. Conventional treatment for ischemic arrhythmia relied mainly on the classic anti-arrhythmic drugs available. However, many patients are still subjected to refractory ischemic arrhythmia [1]. Yang and his colleagues have confirmed that microRNA-1 (miR-1) is a potential anti-arrhythmic target. MicroRNA-1 can relieve arrhythmia via repression of miR-1 [2]. When miR-1 was overexpressed in individuals with coronary artery disease, and normal or myocardium-infracted rat, it would exacerbate arrhythmogenesis. After injected its antisense oligonucleotides (AMO-1) into infracted rat hearts, down-regulation of free miR-1 by these oligonucleotides inhibitors can relieve arrhythmogenesis. In addition, the studies have indicated that overexpression of miR-1 can down-regulate GJA1 (which encodes connexin 43) and KCNJ2 (which encodes the K\textsuperscript{+} channel subunit kir2.1) levels leading to lower conduction and depolarization of the cytoplasmic membrane [3,4].

Gene therapy is a promising strategy for the treatment of ischemic heart diseases [5–8]. With the development of gene...
delivery, it’s feasible to regulate beneficial or pathological proteins expression to achieve desired therapeutic effects [9,10]. Local injection of miRNA analogs into ischemia myocardial tissue can relieve the syndrome of rats suffered ligation of left anterior descending (LAD) [11]. However, it is not practicable to inject therapeutics into myocardium in the clinical. Application of antisense oligonucleotides through parenteral pathway has to face some problems such as degradation by environmental enzymes, low penetration across barriers, poor control of dosage and duration, and off-target effects [12–14]. MicroRNA-1 is considered to be muscle-specific and mainly expressed in adult cardiac and skeletal muscle tissues [15,16]. Hence, the side effects on skeletal muscle should be taken into account when AMO-1 was intravenously administrated to therapy ischemic arrhythmia.

Target strategy is a promising approach for oligonucleotides delivery to pathological regions. It has received increasing benefits, not only for the enhancement of therapeutic efficiency, but also for the reduction of systemic side effects [17,18]. Recently, many groups have designed targeting liposome based on receptor-, or antibody-mediated drug delivery system mechanisms [19–21]. In terms of targeting liposomes, more attentions were paid on maximizing the targeting efficiency and minimizing side effects. Many approaches have been employed to improve targeting efficiency of cationic liposome such as cations modification, cleavable PEG coating, and decoration with cell penetrating peptides [22,23]. The studies have shown that a pathological change in ischemic myocardium is overexpression of cardiac troponin I (cTnI). The similarity for amino acid sequence of troponin between cardiac and skeletal muscle is only about 40% [24]. Cardiac troponin I, as a current gold-standard marker, which is uniquely expressed when only cardiac damage occurs [25]. The cTnI will leak out the cell and maintain the high-level into myocardium tissue, provided that cardiomyocytes die due to tissue hypoxia. The pathological phenomena can be used to design the targeting drug delivery system.

Based on this hypothesis, we designed liposomes modified with anti-cardiac troponin I antibody (anti-cTnI Ab) as AMO-1 carriers. In the present studies, anti-cTnI Ab was conjugated to DSPE-PEG-MAL within the phospholipid bilayer of liposomes. Characterizations of liposomes such as size distribution, zeta potential, entrapment efficiency, and release kinetics behavior in vitro were investigated. Endocytosis by primary myocardial cells and distribution in the cells were observed by live cell station and flow cytometry. The targeting efficiency in ischemic myocardium tissue was assessed by in vivo imaging system. After administration, the level of miR-1 in the ischemic tissue was evaluated by real-time PCR and the electric current of ex vivo myocardial cell was recorded by patch-clamp. In addition, immunohistochemistry method was used to evaluate the pathological leakage of cTnI into the myocardium tissue as a reference of dosage regimen, and the therapeutic improvements of CX43, and Kir2.1 of heart cells.

2. Materials and methods
2.1. Materials

Egg phosphatidylcholine (EPC) and cholesterol (CHOL) were purchased from Bio Life Science & Technology Co., Ltd. (Shanghai, China). Distearylophosphatidyl-ethanolamine (DSPE-PEG2000) was obtained from NOF Corporation (Tokyo, Japan). Rhodamine B (Rhodamine B) and 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine iodide (DiR) were obtained from HEDE Biotechnology Co. Ltd. (Beijing, China). Hoechst 33258 and DIO (3,3-dioctadecylxocarboxylic perchlorate) were purchased from Beijing Fanbo Science & Technology Co., Ltd. (Beijing, China). Rho-miR-1 inhibitor (AMO-1) was purchased from Shanghai GenePharma Co., Ltd. Rabbit Anti-cTnI/Troponin/TNNCI (cTn Ab) was purchased from Beijing Biosynthesis Biotechnology Co., Ltd. Anti-troponin I cardiac muscle Antibody (anti-cTnI Ab), KIR2.1 inhibitor (AMO-1) was purchased from Shanghai GenePharma Co., Ltd. Rabbit Anti-cTnI/Troponin/TNNCI (cTn Ab) was purchased from Beijing Biosynthesis Biotechnology Co., Ltd. Anti-troponin I cardiac muscle Antibody (anti-cTnI Ab), KIR2.1 inhibitor (AMO-1) was purchased from Shanghai GenePharma Co., Ltd. Rabbit Anti-cTnI/Troponin/TNNCI (cTn Ab) was purchased from Beijing Biosynthesis Biotechnology Co., Ltd.

2.2. Preparation of various liposomes (LIP, cT-LIP, A-LIP, cT-A-LIP)

2.2.1. Preparation of liposomes (LIP, A-LIP)

The liposomes, containing EPC, CHOL and DSPE-PEG2000, with molar ratio of 49/50/1, were prepared by the thin film hydration process complied with a method previously reported [26]. Briefly, the lipids were dissolved in anhydrous ethanol in a rotary bottle. A thin lipid film was formed after evaporation under reduced-pressure condition. The dry lipid film was hydrated by physical saline solution (1 mL NaCl per 9.0 mg EPC or AMO-1 solution (3.2 mg AMO-1 per 9.0 mg EPC). The dispersion was dispersed by sonication for 3 min (running 10 s, pause 6 s, 10 times) and then passed through a polycarbonate membrane (220 nm and 150 nm for 3 times in turn) using an extruder. The liposomes samples (LIP and A-LIP) were stored at 4 °C for the further studies. As for DiR liposomes, hydrophobic dye dissolved in anhydrous ethanol followed with similar process as LIP. As for Rhodamine liposomes, hydrophilic dye dissolved in the AMO-1 solution followed with similar process as A-LIP.

2.2.2. Preparation of anti-cTnI Ab modified liposomes (cT-LIP, cT-A-LIP)

Anti-cTnI Ab-DsPE-PEG solution was synthesized by a method previously reported [27]. Briefly, DSPE-PEG (2000)-maleimide (DSPE-PEG-MAL, molecular weight 2900) and anti-cTnI Ab (molecular weight 23KD) were respectively dissolved in a buffer solution (10 m Hepes solution, PH 7.4). The anti-cTnI Ab solution was gently vortexed at an equal volume of DSPE-PEG-MAL solution overnight in a dark tube at 4 °C. Then, anti-cTnI Ab-DsPE-PEG solution was obtained. Next, the anti-cTnI Ab-DsPE-PEG solution was incubated with lipid (prepared by procedure 2.2.1) for 2 h in a dark tube at 37 °C. Then cT-LIP was obtained. Similarly, cT-A-LIP would be obtained after that A-LIP (3.2 mg AMO-1 per 9.0 mg EPC) was incubated with the anti-cTnI Ab-DsPE-PEG solution. As for DiR liposomes, hydrophobic DiR dissolved in anhydrous ethanol followed with similar process as cT-LIP. As for cT-Rho liposomes, hydrophilic dye dissolved in the AMO-1 followed with similar process as cT-A-LIP.

2.3. Characterization of various liposomes

2.3.1. Particle size and zeta potential

The size distributions and morphologies of various liposomes were determined by a dynamic light scattering method (Nano ZS 90, Malvern, atomic force microscopy (Nanoscope III Digital Instruments/Veeco, Santa Barbara) and transmission electronic microscopy (Tecnai G2 F20, STM; FEI, Hillsboro), respectively. The various liposomes were diluted to some extent before measurement, using an appropriate volume of DEPC water. Every sample was measured in triplicate.

The various liposomes were disposed according to the protocol above (2.3.1), using the electrophotochore mobility method (Nano ZS 90, Malvern) at room temperature. Triplicate measurements were done for each sample.

2.3.2. Entrapment efficiency

The entrapment efficiencies of the A-LIP and cT-A-LIP were, the LIP as the negative control, determined according to methods previously reported [28–30]. The liposomes were centrifuged at 20, 000 × g for 30 min; the supernatants were diluted (if necessary) and analyzed for AMO-1 (marked by TAMRA at 510 nm), which contains cTnI and AMO-1 was intravenously administrated as a reference to dosage regimen, and the therapeutic improvements of CX43, and Kir2.1 of heart cells.

2.3.3. In vitro release of A-LIP and cT-A-LIP

The rate of release of cT-A-LIP in vitro was determined using a dynamic dialysis method in phosphate-buffered saline solution (PBS, pH 7.4) [31]. Briefly, all the liquid and containers were treated with DEPC and sterilized. 0.5 mL of cT-A-LIP solution was placed into the dialysis bag with a cutoff 8000–14,000 Da. The liposome solution was then dialyzed against 100 mL of PBS at 37 °C using a magnetic stirring apparatus with a low rotational speed. After 1, 2, 4, 6, 18, 24 and 36 h, 2 mL lysate sample was then collected. The samples were disposed according to the protocol above (2.3.1), using the electrophoretic mobility method (Nano ZS 90, Malvern) at room temperature. Triplicate measurements were done for each sample.
volume of fresh PBS was added. At the last time point, the dialysis bag was cut to mimic the complete release. The optical absorbance of AMO-1 released from dialysis bag was determined using UV–visible spectrophotometer and cumulative drug release (%) was calculated.

2.4. Ex vivo experiments

2.4.1. Primary culture of neonatal myocardial cells
All animals care and experimental procedures were complied with the regulations of the ethics committee of Harbin Medical University. Two-day-old Wistar rat pups were sacrificed after soaking into 75% ethanol solution for 1–2 min and the hearts were rapidly removed, cleaned, minced and triturated into small pieces. Then the ventricular tissues were stored in 10 mL centrifuge tubes and digested by equal volume Trypsin-EDTA solution (provided by Beyotime) for 1 min, the digested solution was transferred to DMEM medium supplemented with 10% fetal bovine serum and stored at 4 °C. The above-mentioned ventricular tissues digestion procedures were repeated until the tissues were disappeared. The collected cells suspension was filtered with 200 mesh screen, centrifuged at 2500 rpm for 3 min, and resuspended in culture medium. Then the myocardial cells suspension was obtained. In the next, myocardial cells were seeded in 6-well plates and incubated at 37 °C, 5% CO2 for 90 min, then refreshed new culture media and incubated for 48 h. The myocardial cells were used for trypan blue staining, time-lapse live cell imaging and flow cytometry.

Fig. 1. Characterizations of anti-cTnI antibody modified liposomes loaded with AMO-1. (A) Particle size. (B) Zeta potential. (C) Transmission electron microscopic image. (D) Representative atomic force microscopic image.

Fig. 2. Cell viability of AMO-1-loaded liposomes in myocardial cells was evaluated by MTT. The concentrations of AMO-1 in liposome preparations were 0.03, 0.125, 0.5, 2.0 and 8.0 μM. Notes: Data were expressed as Mean ± standard deviation (S.D.) for n = 6 (*P < 0.05, **P < 0.01, ***P < 0.001 versus No treatment group).
2.4.2. Liposomal cytotoxicity

The cytotoxic effects of various liposomes were determined as previously described [24,32]. Digestive myocardial cells suspension wasseed in 96-well plates at a density of 2.0 × 10^4 per well and incubated for 48 h. An 8 μM of AMO-1 encapsulated in a corresponding liposomes was diluted to 2, 0.5, 0.125 and 0.03 μM with serum-free culture medium. Then various concentrations of AMO-1 loaded liposomes were added to 96-well plates, every given preparation was added into 6 wells. After 4 h, the cells were added 10 μL of MTT (5 mg/mL) per well and continued to incubate for 4 h at 37 °C. Then the MTT was removed and added 100 μL DMSO per well for dissolving formazan, a production comes from the

![Graph A1: Count vs Log Count](image1)

![Graph A2: Fluorescence Intensity vs Ctl, Free dye, cT-A-LIP, A-LIP](image2)

![Figure B: Uptake and distribution of various Rho-labeled liposomes in cardiocytes using FACS flow cytometry assay (A1, A2) and time-lapse live cell imaging analysis (B). (A1), uptake intensity of the control (red histogram), Rho-labeled cT-A-LIP (green histogram), A-LIP (blue histogram), and free dye (orange histogram). (A2), Statistical analysis of fluorescence intensity of various liposomal preparations. Data were expressed as mean ± S.D., **P < 0.01, ***P < 0.001 versus Ctl. (B), distribution of various liposomes in myocardial cells suffered from hypoxia for 40 min. The cell nuclei was stained with Hoechst 33,258 (blue), cytomembrane with DiO (green), and various liposomes with rhodamine (red), respectively. In LIP and A-LIP groups, there were no differences in accumulation of fluorescence trackers in cell nuclei and cytoplasm. However, to a large extent, cT-LIP and cT-A-LIP were intensively distributed in cytoplasm via specific combination of cTnI (overexpressed in cytoplasm) and anti-cTnI Ab (existed on the surface of liposomes) where microRNA matured. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

![Images B1: Image A](image3)
reaction between MTT and mitochondrial dehydrogenase in the viable cells. The optical density (OD) was measured at 492 nm with an enzyme-linked immunosorbent assay reader (Tecan, Austria).

2.4.3. Flow cytometry analysis

The uptake of Rho-labeled liposomes by primary myocardial cells was measured using an FACS scan flow cytometer (Becton Dickinson FACS Aria I, Mountain view, CA) as previously described[33,34]. The primary cells were seeded in six-well plates at a density of $2 \times 10^5$ cells per well and incubated for 48 h. The primary cells were treated with free Rho, Rho-labeled cT-LIP and Rho-labeled LIP, the DMEM culture medium was used as control group. After 4 h, the cells were detached with 0.25% trypsin, harvested and resuspended in phosphate-buffered solution for flow cytometric assay. The Rho loaded liposomes uptake was measured and the data was analyzed using Flow Jo 7.6 software.

2.4.4. Time-lapse live cell imaging

Myocardial cells were seeded into a particular plate (Applied Precision Issaquah, WA, USA) for 48 h after digestion and then subjected to hypoxia for 40 min. Myocardial cells were stained with Hoechst 33,258 (10 mg/1 mL medium) and DiO (6 mg/1 mL medium) for 5 min to stain nuclei and membranes, respectively. After washing 3 times with PBS, Rho-labeled LIP, Rho-labeled cT-LIP, A-LIP or cT-A-LIP was added into cell plates containing 1 mL new medium. The treated myocardial cells were imaged using a DeltaVision wide-field microscope system (Applied Precision) built on an inverted microscope base. Time-lapse images, up to 30 min in duration with a time point interval of 15 s, were captured with an image size of 512 x 512 pixels. The images were analyzed using DeltaVision softWoRx software (Applied Precision).

2.4.5. Whole-cell patch-clamp recording of I\textsubscript{k1} in myocardial cells

Single ventricular myocardial cells were isolated from the heart of guinea pigs using standard enzymatic digestion techniques, as described before [35,36]. CT-A-LIP and AMO-1 were added to the myocardial cells suspension and incubated for 2 h. Then the cells were placed into a lucid chamber mounted for adherence and superfused with a tyrode solution containing (in mM) 136 NaCl, 5.4 KCl, 1 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 10 HEPES (pH 7.4), and glucose at concentrations as specified. The standard patch-clamp electrodes were prepared from thin-wall glass capillaries with a horizontal puller (Narishige, Tokyo, Japan). It has a tip resistance of megohms when filled with pipette solution containing (in mM) 130 KCl, 5 Mg-ATP, 10 EGTA, and 10 HEPES (pH 7.3). Then currents were recorded using whole-cell patch-clamp with an Axopatch-200B amplifier and pCLAMP 8.0 software (Axon Instruments Inc., Union City, CA, USA) at room temperature as previously reported[37]. I\textsubscript{k1} as a Ba\textsuperscript{2+}-sensitive current was measured using 300 ms steps from −120 mV to 50 mV (10 mV increments) in the presence of holding potential and absence of 0.3 mM BaCl\textsubscript{2}. Data were analyzed with pCLAMP software and plotted as current–voltage curves. Experiments were performed at room temperature.

2.5. In vivo experiments

2.5.1. Surgical model for myocardial infarction

MI model of female adult Wistar rats was induced by ligation of the left anterior descending artery (LAD) under intraperitoneal anesthesia with 10% Chloral hydrate (350 mg/kg body weight). A left thoracotomy was performed to exteriorize the heart. The left anterior descending coronary artery was immediately ligated at approximately 2−3 mm below the junction between the left auricle and pulmonary cone. Then the thorax was rapidly closed and keeping half an hour of ECG recording. Hallmarks of success included the white surface of the heart, the decrease of heartbeat and the elevation of ST segment. In addition, the sham-operated was performed thoracotomy but not ligation. The surgery field was sterilized by iodophor after a successful model.

2.5.2. Immunohistochemistry examination

The pathological changes of cTnI, Kir2.1 and CX43 were evaluated by immunohistochemistry as previously described[38]. Normal rats and MI rats subjected to

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**Fig. 4.** Time-dependent expressions of cTnI in ischemic myocardium for 1−7 days after MI and the effects of CT-A-LIP and AMO-1 on protein expressions of Kir2.1 and CX43 in ischemic myocardium of MI rats. cTnI protein showed the time-dependent expressions of cTnI in 1−7 days after MI. Compared with control group (CtI) and MI group with no treatment group (MI), the expressions of Kir2.1 and CX43 were up-regulated after AMO-1 and CT-A-LIP administration (MI + AMO-1 and MI + CT-A-LIP). The significant changed sites were labeled by black arrows.
Fig. 5. Myocardium-target efficiency of various DiR-labeled liposomes in MI rats. A1, In vivo images of time-dependent whole body imaging in experimental rats after intravenous injection of various preparations: healthy rats were administrated saline (Ctl), MI rats were respectively given equal volume of DiR-labeled liposomes (LIP), DiR-labeled anti-cTnI Ab modified liposomes (cT-LIP) and free dye (DiR), and Sham-operated rats were given equal volume of cT-LIP (sham). MI rats injected with cT-LIP had more robust fluorescence.
1–7 days’ ligation of LAD were prepared. In addition, MI rats intravenously administrated AMO-1 and cT-A-LIP in three days after ligation, were also prepared. After sacrifice, the hearts were taken out and fixed in 4% paraformaldehyde for 24 h. Next, the left ventricle was immediately dissected out and washed with rapidly with physiological saline, then embedded in paraffin and sectioned at 4 µm thick. The paraffin sections were deparaffinized in xylene, hydrated in 100%, 90%, 80%, and 70% of ethanol. The sections were incubated in 3% H2O2 for 10 min at room temperature and citrate buffer solution for 15 min for antigen retrieval in microwave oven with thawing. When the sections were cooled down to room temperature, 5% BSA was added and incubated for 30 min at 37 °C. Subsequently, sections were incubated overnight at 4 °C with primary antibody anti-cTnI (1:100), anti-Kir2.1 (1:100), or anti-connexin 43 (1:100). Then, sections were washed and incubated for 1 h with specific horseradish peroxidase-conjugated secondary antibody (Histostain™–Plus Kits). The sections were visualized with a diaminobenzidine (DAB) and restained with hematoxylin.

2.5.3. In vivo imaging

In vivo optical imaging was performed by Carestream in vivo FX Professional Imaging System equipped with an excitation-pass filter at 720 nm and emission-pass filter at 700 nm as reported [28,38,39]. The protocol of exposure: exposure time of fluorescence was 30 s and X-ray exposure time was 2 min per image. A total of 25 rats were randomly assigned to five groups: control group, 3 MI groups and sham-operated group. Rats were anesthetized with chloral hydrate (350 mg/kg body weight) and immediately placed in a light-tight chamber. After acquisition of a baseline image, MI rats were respectively injected free DiR, DiR-LIP and cT-DiR-LIP via the tail vein. In addition, the Control and Sham-operated groups were respectively injected equal cT-DiR-LIP physiological saline solution and cT-AMO-1 physiological saline, then embedded in paraffin and sectioned in 5 µm. The characterization of the liposomes was shown in terms of particle size, zeta potential, entrapment efficiency and release intensity than other experimental groups. B1, fluorescence intensity in isolated cardiac and skeletal muscle tissues at 6 h after administration. C1, fluorescence intensity of left ventricular sections in all experimental groups including Ctl, LIP, cT-LIP, sham and DiR groups. D1, cardiac sections of cT-LIP group according to body position from right to left are numbered 1, 2, 3, 4, 5, and 6. A2, B2, C2, D2: Detailed quantitative analysis of fluorescence signals from all experimental rats. Signal activity was expressed as p/s/cm²/sr. Data represented the mean ± S.D. (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 respectively compared with cardiac in the groups (Ctl, LIP, cT-LIP, Sham, DiR); #P < 0.05, ##P < 0.01, ###P < 0.001 versus Section 1.
rate (Table 1, Fig. 1). The mean particle size of all the liposome preparations was similar to each other range from 100 nm to 115 nm. The liposomes were slightly electronegative under −3.8 mV. The A-LIP and ct-A-LIP showed high-level AMO-1 entrapment efficiencies of more than 63% by the thin film hydration method. The morphological of ct-A-LIP were of nice round shape and homogeneous (Fig. 1C, D). The rate of release of ct-A-LIP at 1, 6 and 24 h was 6.0%, 9.8%, and 23.1%, respectively. There were no obvious differences between liposomes loaded with dye trackers and liposomes loaded with AMO-1 in the characterization studies.

3.2. Ex vivo experiments

3.2.1. Liposomal cytotoxicity

The cytotoxicity of the liposomes was evaluated with 0.03, 0.125, 0.5, 2.0 and 8.0 μM of AMO-1 in ct-LIP (ct-A-LIP) and LIP (A-LIP). Cell viability in the serum-free culture well was, as a control, set at 100% (Fig. 2 no treatment). As for the relative viability of myocardial cells, there were no obvious changes in the group treated with below 0.5 μM. However, there was a significantly reduction of cell viability in the group treated with above 2 μM. These results showed that 0.5 μM of AMO-1 in liposomes could create maximum therapeutic effect meanwhile keep less cytotoxic.

3.2.2. Flow cytometry assay

Data from myocardial cellular uptake assay showed that the mean fluorescence intensity of Rho were 167.5 for free dye, 241 for A-LIP, 175 for ct-A-LIP and 109.5 for control group (Fig. 3A1, A2). Compared with control group, the data showed that uptakes of A-LIP and ct-A-LIP by myocardial cells in vitro were increased to 220.1% and 159.8% (a little more than free dye) respectively. The results confirmed that the liposomes after modification were able to be internalized by primary cells.

3.2.3. Time-lapse live cell imaging

Time-lapse Live Imaging was used to monitor intracellular distribution of Rho-labeled liposomes with or without anti-cTnI Ab modification. Fig. 3B showed that liposomes with anti-cTnI Ab ligand (ct-LIP, ct-A-LIP) were intensively distributed in cytoplasm where microRNA matured. It may be due to the specific combination of cTnI expressed in cytoplasm and anti-cTnI Ab existed on the surface of liposomes. However, liposomes without anti-cTnI Ab ligand (LIP, A-LIP) distributed both in cell nuclei and cytoplasm randomly. The dynamic internalization process of liposomes in myocardial cells was shown as a movie file (Supplemental 1). The results showed that anti-cTnI Ab modified liposomes were able to enhance accumulation of dye trackers in cytoplasm where they need exist. Docking in the plasma and limitation of delivery in cell nuclei was able to increase the utilization of AMO-1 and improve the therapeutic effects.

3.2.4. Effects of ct-A-LIP on I_{K1} current in myocardial cells

I_{K1} plays an important role in retaining resting membrane potential (RMP) and the late repolarizing phase of the action potential [29]. Previous studies revealed that RMP was depolarized in myocardial cells in MI rats compared with control rats, and that AMO-1 was able to rescue this effect [2]. To evaluate the role of ct-A-LIP on I_{K1} at physiological RMP values, we performed an analysis at a test potential of 90 mV (Fig. 6C). I_{K1} current densities at −90 mV in myocardial cells treated with AMO-1 or ct-A-LIP were increased to −37.59 ± 1.56 or −16.94 ± 2.72 PA/PF⁻¹, respectively. In control group, the density was −7.91 ± 0.26 PA/PF⁻¹. All the results indicated that ct-A-LIP as well as AMO-1 had an effect on repolarized membrane potential.

3.3. In vivo experiments

3.3.1. Time-dependent expressions of cTnI in ischemic myocardium

Cardiac troponin I is a cardiac-specific isoform which would be overexpressed and released into the blood when cardiac damage. The time-dependent expressions of cTnI in MI rats were shown in Fig. 4 (top two lines labeled as cTnI). The differences from immunohistochemistry indicated that expressions of various proteins in the ischemic cardiomyodium were fluctuated with elongation of ischemic time and the expressions of cTnI in ischemic myocardium had significantly increased three day after MI. The results supported that the third day after MI for tail vein injection was the best choice to improve targeting effects of various liposomes.

3.3.2. Expressions of Kir2.1 and CX43 after therapy

The proarrhythmic action of miR-1 was due to down-regulation of Kir2.1 and CX43, while transfection of AMO-1 abolished the down-regulation in ventricular myocardial cells [2]. However, there was no distinctive effect on abolishing the down-regulation of Kir2.1 and CX43 in ischemic myocardium when the MI rats were administrated AMO-1 via tail vein due to the enzyme degradation. On the contrary, ct-A-LIP obviously increased the expressions of Kir2.1 and CX43 in ischemic myocardium compared with MI group. The increased expressions of Kir2.1 and CX43 were nearly to the control group (Fig. 4 bottom two lines labeled as Kir2.1 and CX43). The results revealed that ct-A-LIP were able to up-regulate the expressions of Kir2.1 and CX43 in MI rats and then repressed arrhythmogenesis.

3.3.3. Myocardium targeting efficiency in MI rats

In vivo imaging was performed in MI rats to determine real-time distribution. Fig. 5A1, A2 showed that the group injected with DiR-labeled ct-LIP had a significantly higher accumulation of fluorescence dye compared with other groups. The fluorescence intensity in the heart reached the peak value at 6 h after injection of DiR-labeled ct-LIP. Accordingly, the isolated heart administrated with ct-LIP also had higher in fluorescence intensity than other groups. However, there was extremely low fluorescence signal in isolated skeletal muscle (Fig. 5B1, B2). Further studies revealed that fluorescence intensity of left ventricular sections from ct-LIP group had higher fluorescence signal than other groups administrated LIP, ct-LIP (sham operation group), free DiR and PBS (negative control) (Fig. 5C1, C2). The fluorescence intensity of cardiac sections (1, 2, 3, 4, 5, and 6) administrated with ct-LIP further confirmed that the left ventricular section from left ventricle had higher fluorescence intensity than other sections, and the fluorescence intensity increased as proximity to the ligation increased (Fig. 5D1, D2). Concentration gradient of cTnI in the different sections may be attributed to severity of hypoxia and diffusion limitation of the protein macromolecules. The studies also revealed that drug was able to be carried to ischemic myocardium tissue via intravenous injection within 5 min which was benefit to first aid of acute heart disease (Fig. 5A1).

3.3.4. Electrocardiograms recordings

Cardiac rhythm of healthy or MI rats was measured using a standard lead II ECG. Hallmarks of success for MI model were not only the whitening on the surface of the heart but also the elevation of ST segment in the ECG (Fig. 6A, MI 5 min, MI 3D, MI 4D). Three days after MI, the MI rats were injected ct-A-LIP. 24 h after administration, ECG were recorded a continuous period of 15 min (Fig. 6A, MI 4D, MI 4D + ct-A-LIP). Fig. 6A, MI 4D + ct-A-LIP lane showed that ct-A-LIP lowered ST segment compared with MI 4D and near to normal level. The results confirmed that ct-A-LIP was
able to relieve arrhythmias, which indicated that some AMO-1 was delivered to cardiac infarction tissue by the drug carriers.

3.3.5. Levels of miR-1

MicroRNA-1 is specifically expressed in cardiac and skeletal muscle tissues, and overexpressed when myocardium ischemia. To verify down-regulation of the overexpressed miR-1 in ischemic myocardium by cT-A-LIP, the levels of miR-1 in different groups were measured. The results showed that expression of miR-1 increased 1.7-fold in MI rats, increased 3.6-fold treated with cTc-LIP and elevated 3.2-fold treated with A-LIP compared with control. On the contrary, the overexpression of miR-1 was reversed by cT-A-LIP in MI rats (0.5-fold, Fig. 6B). The results suggested that cT-A-LIP was able to down-regulate the overexpression of miR-1 in myocardium ischemia.

4. Discussion

MicroRNA-1 mainly expressed in adult cardiac and skeletal muscle tissues but overexpressed in myocardium when ischemia. To improve the therapy effects and limit the side effects of gene therapy for ischemic arrhythmia based on down-regulation of miR-1, a drug delivery system for ischemic myocardium is imperative [12,13,40,41]. Lipid-based carriers are often used to enhance cellular uptake of gene therapeutic agents because it is difficult for free gene enter into cells [7]. In this work, cTnI was, as a targeting ligand, used to conjugate the surface of liposomes because it is cardiac-specific isoform and overexpressed when MI. Moreover, PEGylation of the liposomes surface decreases reticuloendothelial system recognition and prolongs the circulation time in vivo [42,43]. Therefore, cT-A-LIP increased the distribution of AMO-1 in ischemic myocardium as well as decreased the existence of the AMO-1 by skeletal muscle. The characteristics of cT-A-LIP included a low negative charge (−2.5 ± 0.6 mV), small size (105 ± 0.5 nm), good entrapment efficiency (63.4 ± 6.0%) and in vitro release rate (23.1 ± 0.95%) at 24 h. Liposomes are mainly composed of phospholipids and cholesterol, which are components of cell membrane. They often exist in the recipe of nontoxic particulates for gene delivery. In this study, the liposomal cytotoxic assay showed that the cytotoxicity was increased with the uptake increase of AMO-1 (keeping a mass ratio 3000:1 with lipids) by myocardial cells and 0.5 μM of AMO-1 in liposomes was able to create maximum therapeutic effects meanwhile keep less cytotoxic effects. The therapeutic effects of various liposomes (0.5 μM AMO-1) were investigated in the following studies.

To evaluate the cellular uptake and distribution of cT-A-LIP in myocardial cells, flow cytometry and time-lapse cell imaging was
used. Liposomes were labeled by rhodamine B. The flow cytometry showed that cT-A-LIP had higher uptake rate than free dye and control group. Compared with A-LIP, the decreased endocytosis by the cells indicated that there were some effects on uptake of carriers after antibody modification. The time-lapse imaging showed that LIP and A-LIP were accumulated randomly in cell nuclei and cytoplasm. However, cT-LIP and cT-A-LIP were intensively distributed in cytoplasm. The results indicated that anti-cTnI Ab modified liposomes were able to deliver drug to cytoplasm where miR-1 matured through the complex of anti-cTnI Ab and cTnI in cytoplasm. In this way, cT-LIP was able to effectively deliver AMO-1 to cardiomyocyte cytoplasm, therein decreasing the levels of free miR-1.

To evaluate the myocardium targeting of cT-LIP in MI rats, live animal imaging was used [44]. Immunochemical assay showed that the expressions of cTnI had a peak in the 3rd day after MI surgery (Fig. 4, top two lines labeled as cTnI). Hence, MI rats were administrated liposomes in the 3rd day after MI surgery in order to gain maximum drug accumulation in myocardium. Six hours after administration, DiR-labeled cT-LIP showed the highest accumulation in heart in vivo. Ex vivo, the fluorescence intensity in heart was also intensively higher than that in skeletal muscle tissue and focused on ischemic myocardium (Fig 5B1, B2). The results further revealed that DiR-labeled cT-LIP was able to be effectively delivered to ischemic myocardium and reduce damage on normal organs by the carriers.

To assess the therapeutic effects of cT-A-LIP on ischemic arrhythmia, Immunohistochemical analysis, ECG recordings, Real-time PCR and Patch-clamp recording of Ik1 in myocardial cells after MI. On the contrary, the Ik1 current density was also intensively higher than that in skeletal muscle tissue and focused on ischemic myocardium (Fig 5B1, B2). The results further revealed that DiR-labeled cT-LIP was able to be effectively delivered to ischemic myocardium and reduce damage on normal organs by the carriers.

To assess the therapeutic effects of cT-A-LIP on ischemic arrhythmia, Immunohistochemical analysis, ECG recordings, Real-time PCR and Patch-clamp recording of Ik1 in myocardial cells were performed. Immunohistochemical results showed that cT-A-LIP was able to up-regulate the expressions of Kir2.1 and CX43 in MI rats and then repress arrhythmogenesis. ECG results showed that cT-A-LIP intensively decreased ST segments in MI rats. MicroRNA-1 is overexpressed in individuals with coronary artery disease, which exacerbates arrhythmogenesis. However, down-regulation of miR-1 by AMO-1 in infracted rat hearts was able to relieve arrhythmogenesis [2]. In this study, the level of miR-1 expression was significantly decreased in RNA samples isolated from MI rats treated with 0.5 μM of cT-A-LIP compared with A-LIP or cT–NC–LIP (Fig. 6B). However, it’s still desired to further explore an appropriate concentration of cT-A-LIP to maintain the range of miR-1 with an acceptable and therapeutic fluctuation. The Ik1 plays a role in maintaining resting membrane potential (RMP) and the late repolarizing phase of the action potential [21,22]. As described before, the Ik1 current density was reduced in rat ventricular myocardial cells after MI. On the contrary, the Ik1 current density was reversed by cT-A-LIP as well as AMO-1 in ex vivo studies. All the results indicated that cT-LIP was able to relieve ischemic arrhythmia.

5. Conclusions

The cT-LIP not only delivered AMO-1 to ischemic myocardium in MI rats, but validated AMO-1 on relieving ischemic arrhythmia by silencing of miR-1 in ischemic myocardium and restoring the depolarized resting membrane potential (RMP) in MI rats. The cT-LIP was a potential targeting drug delivery carrier, which was able to deliver therapeutic agents to the ischemic myocardium tissues.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2013.12.099.

References


