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Research paper

Adipose-derived stem cells combined with Neuregulin-1 delivery systems for heart tissue engineering

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Dedicated to Hans Peter Merkle on the occasion of his 70th birthday

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ABSTRACT

Myocardial infarction (MI) is the leading cause of death worldwide, and extensive research has therefore been performed to find a cure. Neuregulin-1 (NRG) is a growth factor involved in cardiac repair after MI. We previously described how biocompatible and biodegradable microparticles, which are able to release NRG in a sustained manner, represent a valuable approach to avoid problems related to the short half-life after systemic administration of proteins. The effectiveness of this strategy could be improved by combining NRG with several cytokines involved in cardiac regeneration. The present study investigates the potential feasibility of using NRG-releasing particle scaffold combined with adipose-derived stem cells (ADSC) as a multiple growth factor delivery-based tissue engineering strategy for implantation in the infarcted myocardium. NRG-releasing particle scaffolds with a suitable size for intramyocardial implantation were prepared by TROMS. Next, ADSC were adhered to particle scaffolds and their potential for heart administration was assessed in a MI rat model. NRG was successfully encapsulated reaching encapsulation efficiencies of 92.58 ± 3.84%. NRG maintained its biological activity after the microencapsulation process. ADSCs adhered efficiently to particle scaffolds within a few hours. The ADSC-cytokine delivery system developed proved to be compatible with intramyocardial administration in terms of injectability through a 23-gauge needle and tissue response. Interestingly, ADSC-scaffolds were present in the peri-infarted tissue 2 weeks after implantation. This proof of concept study provides important evidence required for future effectiveness studies and for the translation of this approach.

1. Introduction

Cardiovascular diseases cause more than 17 million deaths each year according to the latest report of the World Health Organization (available in http://www.who.int/cardiovascular_diseases), constituting the greatest health risk in western countries [1]. Despite the advances in pharmacological treatment, a major improvement able to repair the massive loss of cardiomyocytes after a myocardial infarction (MI) has not yet been reached, heart transplantation being the only real option for severe cases. Due to this situation, new approaches have been explored in the last few years [2–5]. One of these strategies is the use of growth factors (GF). GF are thought to benefit the damaged heart through direct effects in the myocardium and by stimulating and mobilizing progenitor cells [6]. However, GF administration presents serious limitations due to their short *in vivo* half-life, physical and chemical instability, and the low oral bioavailability of these macromolecules [7]. The use of drug delivery systems (DDSs) that encapsulate GF might overcome these drawbacks. Microparticles (MP), one of these DDS, could protect proteins from degradation and ensure sustained release among time [8]. Recently, our group explored new therapeutic strategies for MI treatment, based on the use of polymeric MP that release different GF involved in cardiac angiogenesis and neovascularization [8–11]. Neuregulin-1 (NRG) deserves special attention in heart regeneration because it is involved in cardiac repair after MI [12]. This protein plays a crucial role in the adult cardiovascular system by inducing sarcomere membrane organization and integrity [13], cell survival [14,15], and angiogenesis [16]. We recently proved that NRG-releasing MP promoted cardiac repair and improved cardiac performance [11]. NRG-releasing MP effectiveness could be improved by combining this protein with several other cytokines involved in cardiac regeneration. This could be achieved by preparing a polymer-based GF delivery system that allows the release of multiple factors [6]. However, to date, GF delivery systems have not demonstrated the ability to deliver cocktail of factors with distinct kinetics [17]. This aspect, besides the limitation that GF dose and timing are crucial for helping regeneration, makes it difficult to co-administer different GFs [6,7]. The

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combination of NRG-releasing MP with cytokines secreted by stem cells (SC), capable of responding to the host environment, opens up a possible solution to that drawback. Moreover, MP possess many features that make them suitable for use as cardiac scaffolds. In particular, they are biodegradable, biocompatible, and non-toxic, and importantly, they can provide structural support for cell survival and differentiation [18–24].

Among the different SC sources, adipose-derived stem cells (ADSCs) have shown promising results in cardiac repair [25–28]. They are good candidates for cell therapy studies because of their easy isolation from the stromal vascular fraction [29–32] and their extensive differentiation potential. In addition, ADSCs are able to secrete angiogenic and/or anti-apoptotic factors [33], such as granulocyte–macrophage colony stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), fibroblast growth factor (bFGF), and transforming growth factor-β (TGF-β) [31].

For all of these reasons, the primary purpose of this work was to investigate the potential feasibility of NRG-releasing particle scaffold combined with ADSC as a multi GF delivery-based tissue engineering strategy for the ischemic heart. To this end, NRG-releasing delivery system was prepared using Total Recirculation One-Machine System (TROMS), a technique based on the multiple emulsion solvent evaporation method which is suitable for the encapsulation of labile molecules like cytokines and GFs [8,34]. We primarily investigated the physical characteristics of the particle scaffold such as morphology or size. Then, NRG-releasing particle scaffolds were combined with ADSC, and flow properties such as dispersability and injectability of the ADSC particle scaffold suspension were analyzed to avoid complications during their administration. The myocardial response to ADSC combined with NRG-releasing particle scaffold was finally evaluated using a MI rat model to ensure safety and biocompatibility requirements.

2. Materials and methods

2.1. Materials

Poly(lactic-co-glycolic acid) (PLGA) with monomer ratio (lactic acid/glycolic acid) of 50:50 Resomer® RG 503H (Mw: 34 kDa) was provided by Boehringer-Ingelheim (Ingelheim, Germany). Polyethylene glycol (PEG; Mw: 400), human serum albumin (HSA), bovine serum albumin (BSA), dimethylsulfoxide (DMSO), carboxymethyl-cellulose, mannitol, polysorbate 80, sodium azide, and rhodamine B isothiocyanate were provided by Sigma–Aldrich (Barcelona, Spain). Dichloromethane and acetone were obtained from Panreac Quimica S.A. (Barcelona, Spain). Poly(vinylalcohol) (PVA) 88% hydrolyzed (Mw: 125,000) was obtained from Polysciences, Inc. (Warington, USA). Collagen I of rat tail 3 mg/mL, Minimum Essential Medium Alpha (α-MEM) Medium, 0.05% Trypsin–EDTA, Heat inactivated Fetal Bovine Serum (FBS), Phosphate-Buffered Saline pH 7.2 (PBS), and Dulbecco's Modified Eagle Medium (DMEM) were provided by Gibco-Invitrogen (Carlsbad, CA, USA). ADSCs were obtained from inguinal adipose tissue of male Sprague–Dawley transgenic rats. H9c2 cells were provided by ATCC. Poly-D-lysine 1 mg/mL (PDL) was provided by Merck-Millipore (Darmstadt, Germany). rh Neuregulin-1b-iso (NRG) was provided by EuroBioSciences (Friesoythe, Germany). 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium (MTS) was purchased from Promega (Madison, USA). Goat polyclonal antihuman NRG-1 antibody (sc-1793) and horseradish-peroxidase-conjugated donkey anti-goat IgG (sc-2020) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Preparation of NRG-releasing particle scaffold

NRG-releasing PLGA particle scaffolds were prepared by the emulsion solvent evaporation method using TROMS as previously described [11] with minor modifications. In order to obtain batches with a defined particle size, the following TROMS parameters were adjusted: pumping flow, recirculation times to obtain both W_1/O and $W_1/O/W_2$ emulsions, and inner diameters of the needles used to prepare the emulsions. Briefly, the organic phase (O) composed of 100 mg of PLGA dissolved in 4 mL of a dichloromethane/acetone mixture (ratio 3:1) was injected into the inner aqueous phase (W_1) containing 200 μg of NRG, 5 mg of HSA, and 5 μL of PEG 400 dissolved in 200 μL of phosphate-buffered saline (PBS pH 7.9). Next, the inner emulsion (W_1/O) was recirculated through the system under a turbulent regime maintained by a pumping flow through a needle. After this homogenization step, the W_1/O emulsion was injected into the outer aqueous phase (W_2) composed of 20 mL of a 0.5% w/v PVA solution. The turbulent injection through a second needle resulted in the formation of a multiple emulsion ($W_1/O/W_2$), which was allowed to circulate through the system to become homogeneous. The multiple emulsion was stirred for 3 h to allow solvent evaporation. Particle scaffolds were washed three times with ultrapure water by consecutive centrifugations at 4 °C (20,000*g*, 10 min). NRG-releasing particle scaffolds were lyophilized for 48 h without cryoprotective agents (Virtis Genesis 12 EL, Gardines, NY). The conditions of freeze drying were −50 °C to +15 °C over 2 days. After complete lyophilization, the vials were sealed under vacuum and stored at −20 °C until use. Unloaded particle scaffolds were prepared in the same manner without adding NRG. For fluorescence-labeled formulation, rhodamine B isothiocyanate (0.5 mg/mL) was added to the inner aqueous phase, and particle scaffolds were prepared as described.

2.3. NRG-releasing PLGA particle scaffold characterization

2.3.1. Particle size analysis

The mean particle size and size distribution were examined by laser diffractometry using a Mastersizer® (Malvern Instruments, Malvern, UK). Particle scaffolds were dispersed in ultrapure water and analyzed under continuous stirring. The average particle size was expressed as the volume mean diameter in micrometers.

2.3.2. Drug content

The amount of NRG encapsulated in the particle scaffold was determined by dissolving 0.5 mg of lyophilized loaded particles in 25 μL of DMSO and was quantified using Western blot. After electrophoresis and transference, the membranes were blocked with 5% nonfat dried milk in TBS plus 0.05% Tween 20 for 2 h, then incubated overnight at 4 °C with primary antibody goat IgG-NRG-1β-IGGF2 (sc-1793) 1:50. After several washes the membranes incubated with anti-goat IG-HRP (sc-2020) 1:2000 secondary antibody for 2 h. Immunoreactive bands were, after several washes, visualized using LumiLight plus Western blotting substrate (Roche Diagnostics, Mannheim, Germany). The quantifications were determined by ImageQuant RT ECL. Sample values were quantified using a blotting standard curve with known amounts of NRG.

2.3.3. In vitro bioactivity assay

The bioactivity of NRG released from particle scaffolds was evaluated *in vitro* by determining the proliferative capacity of H9c2 cells after NRG treatment. H9c2 cells obtained from embryonic BD1X rat heart tissue were cultured in DMEM medium supplemented with 10% FBS, 1% glutamine, and 1% penicillin/streptomycin at 37 °C under 5% CO_2/95% air [35–37]. Cells were subcultured when 60% confluency was achieved. In order to quantify cell proliferation after NRG stimulation, cells were seeded in 96-well tissue