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Transplantation of adipose-derived stem cells combined with neuregulin-microparticles promotes efficient cardiac repair in a rat myocardial infarction model

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ABSTRACT

Tissue engineering is a promising strategy to promote heart regeneration after a myocardial infarction (MI). In this study, we investigated the reparative potential of a system that combines adipose-derived stem cells (ADSCs) with microparticles (MPs) loaded with neuregulin (NRG), named ADSC-NRG-MPs, on a rat MI model. First, cells were attached to the surface of MPs encapsulating NRG and coated with a 1:1 mixture of collagen and poly-D-lysine. One week after in vivo administration, the system favored the shift of macrophage expression from a pro-inflammatory to a regenerative phenotype. At long-term, the adhesion of ADSCs to MPs resulted in an increased cell engraftment, with cells being detectable in the tissue up to three months. In consonance, better tissue repair was observed in the animals treated with cells attached to MPs, which presented thicker left ventricles than the animals treated with ADSCs alone. Moreover, the presence of NRG in the system promoted a more complete regeneration, reducing the infarct size and stimulating cardiomyocyte proliferation. Regarding vasculogenesis, the presence of ADSCs and NRG-MPs alone stimulated vessel formation when compared to the control group, but the combination of both induced the largest vasculogenic effect, promoting the formation of both arterioles and capillaries. Importantly, only when ADSCs were administered adhered to MPs, they were incorporated into newly formed vessels. Collectively, these findings demonstrate that the combination of ADSCs, MPs and NRG favored a synergy for inducing a greater and more complete improvement in heart regeneration and provided strong evidence to move forward with preclinical studies with this strategy.

1. Introduction

Tissue engineering (TE) is a promising strategy for the regeneration of damaged tissues. The combination of stem cells and growth factors (GFs) with a biomaterial scaffold [1] has been demonstrated to protect GFs from rapid degradation [2] and to provide three-dimensional support that favors cell engraftment and survival [3]. Moreover, the combination of GFs and stem cells increases the possibility of activating different pathways to promote tissue repair [4,5]. All this considered, TE seems to be a promising therapy in heart damage [6,7]. Within cardiovascular diseases, myocardial infarction (MI) is the most frequent, causing millions of deaths per year worldwide according to the World Health Organization [8]. After a MI, the heart is dramatically damaged and to date, there is no treatment available to repair or reduce the massive loss of cardiomyocytes. Accumulative research evidence indicates beneficial effects of adipose-derived stem cells (ADSCs) for treating MI in both animal models and humans, even though low cell survival and engraftment have been observed [9–11]. ADSCs are easily isolated from adipose tissue, grow fast in culture media and share common properties with bone marrow stem cells in terms of multipotency and immunoregulatory properties [12,13]. ADSCs implantation participates in the repair of the damaged cardiac muscle by inducing angiogenesis, mainly due to a paracrine effect in the infarcted area [14,15]. These adult stem cells are able to secrete various GFs, such as vascular endothelial growth factor and hepatocyte growth factor, among others [16,17]. As the secretion of GFs by cells is regulated by tissue microenvironment signals, the concentration of secreted GFs is in the physiological range and can be adapted according to the requirements of the different stages of heart healing [18]. Although some studies have already reported the efficacy of ADSCs in cardiac regeneration [9,10], the possibility of

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combining them with GFs different from those secreted by those cells, such as neuregulin-1 (NRG), may induce a better regenerative response. NRG is a GF that plays a crucial role in the adult cardiovascular system [19] by inducing sarcomere membrane organization and integrity, cell survival, cardiomyocyte proliferation and angiogenesis [20–22]. Our group has recently shown that microparticles (MPs) allow controlled delivery of therapeutic proteins like NRG in the MI region, accompanied by a significant improvement in cardiac function in both rat and pig models of MI [23–25]. We have also demonstrated that NRG-MPs combined with ADSCs, known as ADSC-NRG-MPs, are totally biocompatible with infarcted rat hearts [26]. Based on these results, in the present study we sought to enhance ADSCs survival in the injured myocardium of a rat MI model and likewise to improve tissue repair by the adhesion of the cells to NRG-MPs. The potential reparative activity of ADSCs and NRG-MPs, alone or in combination, was first investigated. We then determined cell survival and cardiac differentiation. Finally, the interactions between MPs and ADSCs with the macrophages of the innate immune system were examined to determine whether a shift to regenerative macrophages was induced. Collectively, the results obtained indicate that the use of NRG-MPs combined with ADSCs led to increased cell engraftment, thus improving treatment efficacy and providing a rationale for the future application of this technique in clinical studies.

2. Material and methods

2.1. Materials

Poly(lactic-*co*-glycolic acid) (PLGA) with a 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, dimethylsulfoxide, carboxymethyl-cellulose, mannitol, polysorbate 80, sodium azide, sigmacote (SL2) and monoclonal anti-actin α-smooth muscle-Cy3 antibody (C6198) were provided by Sigma-Aldrich (Barcelona, Spain). Dichloromethane was obtained from Panreac Quimica S.A. (Barcelona, Spain). Poly(vinyl alcohol) (PVA) 88% hydrolyzed (Mw: 125,000) was obtained from Polysciences, Inc. (Warrington, PA, 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) and Phosphate Buffered Saline pH 7.2 (PBS) were provided by Gibco-Invitrogen (Carlsbad, CA, USA). ADSCs were obtained from inguinal adipose tissue of male Sprague-Dawley transgenic rats. H9c2 cells were obtained from ATCC. Poly-D-Lysine (PDL) 1 mg/mL was obtained from Merck-Millipore (Darmstadt, Germany) and recombinant human Neuregulin-1b-iso (NRG) by EuroBioSciences (Friesoythe, Germany). 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium (MTS) was purchased from Promega (Madison, WI, USA). Goat polyclonal anti-human 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). Rabbit polyclonal anti-GFP antibody (ab290), chicken polyclonal anti-GFP (ab13970), mouse monoclonal anti-cardiac troponin-T antibody [1C11] (ab8295) and rabbit monoclonal [Y59] anti-CCR7 (ab32527) were supplied by Abcam (Cambridge, UK). Alexa Fluor 488 goat anti-rabbit (A11008), Alexa Fluor 594 goat anti-mouse (A11032), Alexa Fluor 594 goat anti-rabbit (A11012), Alexa Fluor 488 goat anti-chicken (A11039) and DAPI nucleic acid stain (D1306) were supplied by Molecular Probes-Invitrogen (Carlsbad, CA, USA). Mouse monoclonal anti-CD163 antibody (MCA342R) was provided by Bio-Rad AbD Serotec (Raleigh, NC, USA). Goat serum (X0907) was provided by Dako (Barcelona, Spain). Donkey anti-rabbit FITC (711-096-152) was provided by Jackson ImmunoResearch (West Grove, PA, USA). Rabbit anti-caveolin-1 antibody (#3238) was provided by Cell Signaling Technology (Danvers, MA, USA). Alexa Fluor 647 mouse anti-human Ki-67 (558615) was provided by BD Pharmingen (San Jose, CA, USA).

2.2. Preparation of NRG-releasing particles

NRG-releasing PLGA particles were prepared by a multiple emulsion solvent evaporation method using a total recirculation one-machine system (TROMS) as previously described [26]. 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 PBS. 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. MPs were washed three times with ultrapure water by consecutive centrifugations at 4 °C (20,000*g*, 10 min) and lyophilized for 48 h without cryoprotective agents (Virtis Genesis 12 EL, Gardiner, NY, USA). Unloaded MPs were formulated without adding NRG.

2.3. Characterization of NRG particles

Particle size and size distribution were measured by laser diffractometry using a Mastersizer® (Malvern Instruments, Malvern, UK). Particles were dispersed in ultrapure water and analyzed under continuous stirring. The average particle size was expressed as the volume mean diameter in micrometers. Particle surface charge was determined by zeta potential measurement using ZetaPlus®, based on the analysis of complete electrophoretic mobility distributions (Brookhaven Instruments Corp., NY, USA). The morphology of MPs was analyzed by scanning electron microscope (SEM, Philips XL 30 ESEM-FEG). Briefly, 0.1 mg of MPs were resuspended in 50 μL of deionized water and sputtered with a thin metallic layer before analysis. Encapsulation efficiency was studied by western blot assay, as described elsewhere [26]. The bioactivity of MP-released proteins was evaluated in vitro by determining H9c2 proliferative capacity following GF treatment by MTS assay as previously described [26].

2.4. Particle surface modification

In order to favor cell attachment to the particle surface, MPs were overlaid with 0.1 or 0.5 μg/cm^2 of collagen type I or PDL alone, or 0.5 μg/cm^2 of a 1:1 mixture of both. Particle coating was performed in 15 mL sigmacote falcon tubes. MPs were re-dispersed in acidified PBS (pH 5.7) and the mixture was sonicated until the particles were completely dispersed in the solution. Then coating molecules were added to the falcon tube and mixed with the particles under rotation at room temperature for 60 min. Coated particles were washed with distilled sterile water and lyophilized for long term storage without a cryoprotectant. Zeta potential and morphology of coated MPs were examined as described in Section 2.3.

2.5. Isolation and culture of ADSCs

ADSCs were obtained by in vitro culture of the stromal vascular fraction isolated from inguinal adipose tissue of 5 male Sprague–Dawley transgenic rats that expressed the green fluorescent protein (GFP) as previously described [9]. A homogeneous population of ADSCs was cultured in α-MEM medium supplemented with 10% FBS, 1 ng/mL bFGF and 1% penicillin/streptomycin. Cells were sub-cultured when 80% confluence was reached. The percentage of ADSCs that expressed GFP was assessed by flow cytometry.