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The contribution of stem cell therapy to skeletal muscle remodeling in heart failure

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ABSTRACT

Background: The aim of our study was to investigate whether stem cell (SC) therapy with human amniotic fluid stem cells (hAFS, fetal stem cells) and rat adipose tissue stromal vascular fraction cells–GFP positive cells (rSVC-GFP) was able to produce favorable effects on skeletal muscle (SM) remodeling in a well-established rat model of right heart failure (RHF).

Methods: RHF was induced by monocrotaline (MCT) in Sprague–Dawley rats. Three weeks later, four millions of hAFS or rSVC-GFP cells were injected via tail vein. SM remodeling was assessed by Soleus muscle fiber cross sectional area (CSA), myocyte apoptosis, myosin heavy chain (MHC) composition, satellite cells pattern, and SC immunohistochemistry.

Results: hAFS and rSVC-GFP injection produced significant SC homing in Soleus (0.68 ± 1.0 and 0.67 ± 0.75% respectively), with a 50% differentiation toward smooth muscle and endothelial cells. Pro-inflammatory cytokines were down regulated to levels similar to those of controls.

SC-treated (SCT) rats showed increased CSA ($p<0.004$ vs MCT) similarly to controls with a reshift toward the slow MHC1 isoform. Apoptosis was significantly decreased ($11.12. \pm 8.8$ cells/mm^3 hAFS and $13.1 + 7.6$ rSVC-GFP) ($p<0.001$ vs MCT) and similar to controls (5.38 ± 3.0 cells/mm^3).

RHF rats showed a dramatic reduction of satellite cells(MCT 0.2 ± 0.06% Pax7 native vs controls 2.60 ± 2.46%, $p<0.001$), while SCT induced a repopulation of both native and SC derived satellite cells ($p<0.005$).

Conclusions: SC treatment led to SM remodeling with satellite cell repopulation, decreased atrophy and apoptosis. Modulation of the cytokine milieu might play a crucial pathophysiological role with a possible scenario for autologous transplantation of SC in pts with CHF myopathy.

1. Introduction

Chronic heart failure (CHF) is a clinical syndrome characterized by decreased exercise capacity with symptoms such as dyspnea and fatigue [1].

Alteration of skeletal muscle (SM) in terms of muscle wastage, transition from slow oxidative to fast anaerobic fibers [2,3] and ergoreceptor dysfunction [1,4] can contribute to further reduce exercise capacity. An inflammatory status with increased levels of circulating pro-inflammatory cytokines, neuroendocrine activation and catabolic/anabolic imbalance plays an important role in this syndrome [5–7]. In fact inflammation by itself is able to produce ubiquitin-dependent wastage, alteration of AKT-FOXO signaling with inadequate growth [8], apoptosis [9] and impaired regeneration [10,11]. These physiopathological conditions can be modified by treatment with ACEIs or ARBs [12,13], physical training [14] and drugs able to block pro-apoptotic signaling [15–17].

Randomized and non-randomized clinical trials with a variety of stem cells (SC), including bone marrow (BM)-derived cells, endothelial precursor cells (EPCs), skeletal myoblasts and mesenchymal stem cells (MSC), have shown controversial results in the heart of HF patients, but nothing is known of regenerative therapy in SM of CHF [18–20].

The beneficial effects of SC have been attributed to many factors varying from paracrine response, such as cytoprotective effects modulated by cytokines, to neovascularization with endothelial cell differentiation [18], proangiogenic and proarteriogenic effects [19], rather than differentiation into new parenchymal cells [10,11,21,22].

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Whether SC treatment could induce favorable changes in the SM needs to be demonstrated and the mechanism by which these changes occur need to be elucidated.

The aim of this study was to evaluate in a rat model of RHF, mimicking the human HF syndrome [23], SC engraftment and differentiation into skeletal myocytes and satellite cells. The paracrine, pro and anti-inflammatory response and apoptosis, along with qualitative and quantitative changes of skeletal muscle were also studied. We compared two different populations of SC (rat adipose tissue stromal vascular fraction GFP positive cells (rSVC-GFP) [24] and c-Kit selected human amniotic fluid stem cells (hAFS) [25].

2. Materials and methods

2.1. Animals and development of RV hypertrophy and failure

Right sided heart failure (RHF) was induced in male Sprague–Dawley rats, weighting 90 to 100 g, by injecting intraperitoneally 30 mg/Kg MCT according to Vescovo et al. [13,15]. MCT is a well established model of RHF which mimics the CHF syndrome in man [13,15]. After 21 days, when pulmonary hypertension (PH) was present and RV hypertrophy and RHF have developed, rats were randomly separated into five different groups:

a) 10 MCT rats, with PH and RHF, treated with saline injected into the tail vein.
b) 7 MCT rats injected with hAFS, harvested from human amniocentesis samples.
c) 11 MCT rats injected with rSVC-GFP, vascular progenitor cells, harvested from adipose tissue of GFP rats.
d) 5 MCT rats, injected with DMEM, which served as control for the SC suspension milieu.
e) 5 untreated rats, injected with saline only, that served as controls.

All the five groups received treatment with cyclosporine (10 mg/kg/die) and azithromycin in drinking water, which was started at day 21, after MCT administration.

After seven days (28 from MCT injection) all animals were killed. Hearts and Soleus muscles were excised and frozen in liquid nitrogen or paraformaldehyde-fixed and paraffin-embedded.

Experiments were approved by the University of Padua Biological Ethical Committee and the Investigation conforms to the Guide for Care and Use of Laboratory Animals published in 1996 by the US National Institute of Health.

2.2. Isolation, culture and injection of hAFS cells

Human AFS c-Kit selected (hAFS) cells were derived according to De Coppi et al. [25], and Chiavegato et al. [26]. Samples of amniotic fluid cells (AF) were collected by amniocentesis mean gestational age 12 weeks, during routine prenatal diagnosis. A written consensus was obtained from each woman to use the AF experimentally.

Cytogenetic analysis revealed normal karyotype. AF were diluted with PBS pH 7,2 (1:2 v/v) and then spun at 1200 rpm in a Heraeus Multifuge S-R (Milan, Italy). Pellets were re-suspended in the Amniotic Culture Medium (ACM; 63% αMEM (Invitrogen, Milan, Italy), 20% of Chang Medium (Chang B plus Chang C; Irvine Scientific, Santa Ana, CA), 15% of fetal bovine serum containing streptomycin, penicillin and L-glutamine). Cell seeding was performed in non-tissue culture Petri dishes. After 3 days, non-adherent cells and debris were discarded and the adherent cells cultivated in pre-confluency. Adherent cells were detached from the plastic plate using a trypsin–Na–EDTA solution (Invitrogen, Milan, Italy), and then immuno-magnetically selected for c-Kit using a monoclonal anti-c-Kit (CD117) antibody (Santa Cruz, Santa Cruz, CA) and a goat IgG anti-mouse IgG (Dynabeads M-450 (Miltenyi Biotech, Bergisch Gladbach, Germany). Control cell sorting was performed by non immune IgG, followed by the secondary antibody. c-Kit + cells were expanded and subsequently cloned by limiting dilution and kept growing in sub-confluent conditions in the presence of ACM. These cells showed self renewal and multilineage potential as already described [25–27].

2.2.1. Cytofluorimetric analysis of hAFS cells

Human AFS cells were incubated with anti human antibodies: CD29-FITC, CD44-FITC, CD73-PE, CD90-FITC, CD105 PE, CD80 FITC and CD86-PE (Beckton Dickinson, Pharmingen, San Jose, CA), SSEA-4 FITC (Santa Cruz). Cells have been analyzed for HLA-ABC FITC and HLA-DR PE (Immunotech, Marseille, France).

Analysis was performed by a COULTER Epics XL-MCL cytometer (Beckman Coulter, CA) and data were elaborated by means of EXPO™ 32 ADC software.

After 5–6 passages 4×10^6 cells were injected [25].

2.3. Isolation and injection of rSVC-GFP positive cells

The stromal vascular fraction was isolated from subcutaneous adipose tissue of 3-months-old GFP + transgenic Wistar rats upon collagenase type II digestion (1 mg/ml) (Sigma-Aldrich) in DMEM at 37 °C for at least 1 h. The sedimented stromal vascular cells (rSVC) obtained by 350 g centrifugation were re-suspended in an erythrocyte-lysing buffer for 5 min and then washed two times in DMEM [24,28].

2.3.1. Cytofluorimetric analysis of rSVC-GFP positive cells

Rat SVC-GFP positive cells were incubated with CD29-FITC, CD31-PE, Cd34-PE, CD44-FITC, CD45-PE, and CD73-PE, purchased from BD and Biolegend.

Analysis was performed by a COULTER Epics XL-MCL cytometer (Beckman Coulter, CA) and data were elaborated by means of EXPO™ 32 ADC Software.

4×10^6 cells in 500 μl DMEM were injected via tail vein in the recipient rats as described above.

2.4. Assessment of RV hypertrophy and failure

In order to make sure that the MCT animals developed RHF, beyond the well known post-mortem signs such as pericardial, pleural and peritoneal effusions, the following measurements were taken:

- Right ventricle mass/left ventricle mass (RVM/LVM) (index of hypertrophy)
- Right ventricular mass/right ventricular volume (RVM/RVV) (index of dilatation) [13].

These two latter indices were calculated with a validated procedure currently adopted in our laboratory, using a computer-based image analyzer system consisting of an Olympus BH2 optical microscope connected to a computer via a video camera (JVC 3-CCD, Yokohama, Japan) and software for image analysis (Image PRO-Plus 4.0; Media Cybernetics, Silver Spring, MA), on formaline fixed transverse sections of the heart taken in the middle portion of the ventricles [13,15].

2.5. Brain natriuretic peptide (BNP) assessment

BNP (brain natriuretic peptide) was measured on sera with an Elisa kit from Phoenix Pharmaceutical, Inc., Burlingame, Ca, USA, following the manufacturer's instructions. The antibody was specific for rat BNP.

2.6. Immunohistochemistry and double-immunofluorescence study

Soleus muscle cryosections and paraffin-embedded sections (5 μm thick) were prepared, treated, and stained for immunohistochemistry according to standard procedures [23,29].

The antibodies used were GFP-specific antibody (Abcam, Prodotti Gianni, Milan, Italy), alfa human mitochondria antibody (Thermo Scientific, USA) coupled with c-Kit (DakoCytomation, Glostrup, Germany), anti Oct-4 stemness marker (Chemicon, Prodotti Gianni, Milan, Italy), alfa smooth muscle actin (DakoCytomation, Glostrup, Germany), alfa von Willebrand factor (Chemicon, Prodotti Gianni, Milan, Italy), CD45 (common leucocytes cell antigen Chemicon, Prodotti Gianni, Milan, Italy), CD68 (macrophages, Chemicon, Prodotti Gianni, Milan, Italy), anti laminin (Sigma-Aldrich, Milan, Italy), and anti slow muscle myosin (Chemicon, Prodotti Gianni, Milan, Italy). Bound antibodies were visualized with anti-mouse or anti-rabbit FITC or rhodamine (TRITC)-conjugated secondary antibodies (Chemicon, Millipore, Prodotti Gianni, Milan, Italy). Nuclei were stained with TO-PRO-3 (Invitrogen, Molecular Probes, Eugene, OR). Micrographs were taken using a laser scanner confocal microscope (Model TCS-SL; Leica, Germany). Samples were also taken from spleen and rSVC-GFP and hAFS cells counted to exclude excessive hemocatheresis.

2.7. Satellite cell identification

Soleus muscle cryosections and paraffin-embedded sections (5 μm thick) were prepared, treated and stained for immunohistochemistry according to standard procedures [23,29]. The antibodies used were GFP-specific antibody (Abcam, Prodotti Gianni, Milan, Italy), alfa human mitochondria antibody (Thermo Scientific, USA), coupled with Pax7 (Hybridoma Bank, University of Iowa, USA) and anti MyoD (Santa Cruz Biotechnology, CA, USA), which are specific for quiescent satellite and activated cells, respectively [30]. Bound antibodies were visualized with anti-mouse or anti-rabbit fluorescein isothiocyanate (FICT) or rhodamine (TRICT) conjugated secondary antibodies (Chemicon Millipore, MA, USA). Nuclei were stained with TO-PRO 3 (Invitrogen, Molecular Probes, CA, USA). Microphotographs were taken using a TCS-SL laser scanner confocal microscope (Leica, Germany).

Three sections per animal and 12 randomly chosen fields per section were evaluated. Satellite cells were identified as mononucleated cells located between the sarcolemma and the basal lamina of muscle fibers. These were counted after laminin staining on the specimens of Soleus of the five groups of animals (magnification 250×). Satellite cell number was expressed as percentage of total myocyte nuclei per mm^2 [23].

2.8. Assessment of cell engraftment and differentiation

Similarly hAFS and rSVC-GFP co-expressing alfa SMA, vWf and myosin positive cells were counted by two independent pathologists on high-power images. Three sections per animal and 12 randomly chosen fields per section were evaluated. Cell number was expressed either as percentage or total number per mm^2.

2.9. Assessment of apoptosis

In situ nick-end labeling (TUNEL) of fragmented DNA was performed on cryo-sections of the Soleus muscle, using the In Situ Cell Death Detection Kit, POD (Roche Diagnostic Applied Science, Milan, Italy).