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SHORT COMMUNICATION

Human cardiac tissue induces transdifferentiation of adult stem cells towards cardiomyocytes

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Abstract

Background aims. The goal was to induce the transdifferentiation (or conversion) of human adipose-derived stem cells to cardiomyocytes using an intracellular extract obtained from adult human heart tissue. *Methods.* Human adult stem cells from lipoaspirates were transiently permeabilized, exposed to human atrial extracts and allowed to recover in culture. *Results.* After 21 days, the cells acquired a cardiomyocyte phenotype, as demonstrated by morphologic changes (appearance of binucleate, striated cells and branching fibers), immunofluorescence detection of cardiac-specific markers (connexin-43, sarcomeric α-actinin, cardiac troponin I and T, and desmin) and the presence of cardiomyocyte-related genes analyzed by reverse transcription–polymerase chain reaction (cardiac myosin light chain 1, α-cardiac actin, cardiac troponin T and cardiac β-myosin). *Conclusions.* We have demonstrated for the first time that adult cardiomyocytes obtained from human donors retain the capacity to induce cardiomyocyte differentiation of mesenchymal stromal cells. The use of autologous extracts for reprogramming adult stem cells may have potential therapeutic implications for treating heart disease.

Key Words: *adipose stem cells, cardiomyogenic differentiation, cell extract, transdifferentiation*

Introduction

Myocardial infarction results in reduced cardiac function because of cardiomyocyte death (1). As the proliferative potential of the terminally differentiated cardiomyocytes is low, the heart is unable to repair itself and after damage non-functional scar tissue is formed. Although the existence of cardiac stem cells has been suggested, their capacity for repair is inadequate to restore normal cardiac function (2). One alternative is to replace damaged or diseased cardiac tissue with cardiomyocytes derived exogenously. Three possible cell sources have been considered for obtaining cardiomyocytes: from human embryonic stem cells by recapitulating the normal sequence of developmental events that leads to cardiomyocyte differentiation, from putative cardiac stem cells by differentiation, and from non-cardiac cells by transdifferentiation (which is defined as the conversion of one cell type to another) (3). Human adipose-derived stem cells (hASC) may represent a potential source for cell therapy. These cells have a number of advantages: (i) they can be isolated from human lipoaspirates with minimal risk or inconvenience to the patient, (ii) the cells can be expanded in culture and (iii) the cells demonstrate the ability to differentiate into different cell types (e.g. adipocytes, chondrocytes, osteocytes and neurons) (4).

It has been shown previously that extracts derived from one differentiated somatic cell type can alter the phenotype of another differentiated cell type, presumably by promoting the uptake of transcription factors and inducing cell-specific gene expression (5). We used

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this approach to induce cardiomyocyte differentiation of hASC. We show for the first time that hASC can be induced to express cardiomyocyte properties following exposure to extract from human heart tissue.

Methods

Isolation and culture of hASC from human adipose tissue

Subcutaneous adipose tissue was obtained from patients by a minimally invasive procedure after informed consent from the patients and approval from the Ethics Committee of the Clinical University Hospital of Málaga, Spain. Isolation and culture of hASC were performed as described previously (6).

Characterization of hASC

The immunophenotype of cultured hASC was analyzed by flow cytometry. Cells were trypsinized, washed and resuspended in phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA; Sigma, St Louis, MO, USA). A total of 2×10^5 cells was incubated in the dark for 30 minwith fluorochrome-conjugated monoclonal antibodies (CD34, CD45, CD90, CD 73, CD105, CD133 and KDR; BD Biosciences, San Jose, CA, USA), washed in PBS and analyzed in a FACSCanto II cytometer (BD Biosciences).

Differentiation assays of hASC

Human ASC were plated at 2×10^3 cells/cm^2 in Dulbecco's modified Eagle's medium (DMEM; Sigma) containing 10% fetal bovine serum (FBS) and allowed to adhere for 24 h. The culture medium was then replaced with specific differentiation inductive media. For adipogenic and osteogenic differentiation, cells were cultured in Adipogenic MSCs Differentiation BulletKit and Osteogenic MSCs Differentiation BulletKit (Lonza, Basel, Switzerland), respectively, for 2 weeks. For chondrogenic differentiation, cells were cultured in NH ChondroDiff Medium (Miltenyi Biotec, Auburn, CA, USA) for 3 weeks. Differentiated cell cultures were stained with Oil red O (Amresco, Solon, OH, USA) for adipogenic differentiation, Alizarin red (Lonza) for osteogenic differentiation and toluidine blue (Sigma) for chondrogenic differentiation.

Preparation of atrial extract and exposure to hASC

Atrial tissue samples were collected from consenting patients undergoing coronary artery bypass surgery, with approval from the Ethics Committee of the Clinical University Hospital of Málaga. Samples were processed just after extraction from the patients, cut into pieces and placed in pre-warmed enzymatic solution (0.15% type II collagenase, 0.25% trypsin, 0.02% glucose in PBS; Sigma) and then incubated for 10 min at 37°C with continuous shaking. Cardiomyocytes were dispersed by forcing the tissue pieces through a 300-μm nylon net filter (Millipore, Billerica, MA, USA). Cardiomyocyte extract preparation and extract exposure to hASC were performed as described elsewhere (7). Briefly, isolated cardiomyocytes were washed twice in cold PBS and then in cold cell lysis buffer (50 mM NaCl, 5 mM $MgCl_2$, 100 mM HEPES, pH 8.2, 1 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride; Sigma). Cells were centrifuged at 800 ***g***, resuspended in 1.5 volumes of cell lysis buffer containing protease inhibitor cocktail (Sigma) and allowed to swell on ice for 45 min The cells were then homogenized by pulse-sonication and the lysate sedimented at 15 000 ***g*** for 15 min at 4°C. The supernatant was collected and used fresh.

For exposure to cell extract, hASC were harvested by trypsin digestion and washed twice in ice-cold PBS and once in ice-cold Hanks' balanced salt solution (HBSS; Gibco-BRL, Paisley, UK). Cells were pelleted at 500 000 cells/reaction in 1.5-mL tubes and suspended in 488 μL ice-cold HBSS, placed in a water bath at 37°C for 2 min and a final concentration of 230 ng/mL Streptolysin O (SLO; Sigma) added. Samples were incubated for 50 min at 37°C. Cells were centrifuged at 300 ***g*** for 5 min at 4°C in a swing-out rotor. The supernatant was removed and 500 μL extract containing 25 μL 50 mM Adenosine Triphosphate (ATP) (Sigma), 2.5 mM Guanosine Triphosphate (GTP) (Sigma), 1.25 mg/mL creatine kinase (Sigma), 0.5 μ phosphocreatine (Sigma) and 20 μL 1mmol/L of each nucleotide triphosphate set (Roche, Indianapolis, USA) added. Cells were incubated with the extracts for 1 h at 37°C. DMEM/10% fetal calf serum (FCS) containing 2 mmol/L $CaCl_2$ was added and the cells were then transferred to 35-mM dishes containing coverslips. After 5 h dead (floating) cells and the Ca^{2+} -containing medium were removed and replaced with fresh DMEM containing 10% FCS and antibiotics; cells were then cultured until use. Control cells were either non-permeabilized and non-extract exposed cells or permeabilized cells not exposed to the extracts.

Immunofluorescence

Cells were washed three times with PBS and fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. Following, cells were permeabilized with 0.1% Triton X-100 for 15 min washed three times with PBS and blocked in 2% blocking buffer solution (Roche) for 1 h at room temperature. Cells were then incubated overnight in primary antibody diluted 1:100 in blocking buffer solution at 4°C, washed three times in PBS and incubated

for 2 h with secondary (Fluorescein Isothiocyanate (FITC)- or Tetramethyl Rhodamine Isothiocyanate (TRITC)-conjugated) antibodies diluted 1:200 in blocking buffer solution. Afterwards, they were washed three times in PBS and the coverslips mounted on slides with DAPI-containing mounting solution (Ultra Cruz™ mounting medium; Santa Cruz, CA, USA). Controls were performed with non-treated cells. Antibodies used were desmin (mouse monoclonal; Sigma), human cardiac-specific troponin I (mouse monoclonal; Research Diagnostics, Flanders, NJ, USA), human cardiac-specific troponin T (rabbit polyclonal; Abcam, Cambridge, UK), connexin 43 (Cx43; goat polyclonal; Santa Cruz) and sarcomeric α-actinin (mouse monoclonal; Sigma). Photographs were taken with a Leica DM 5500B (Solms, Germany) fluorescent microscope, software Meta Systems Isis. Figures were processed with Adobe Photoshop 7.0.

Reverse transcription–polymerase chain reaction

For reverse transcription–polymerase chain reaction (RT-PCR) analysis of cardiomyogenic gene expression, total RNA from treated and non-treated hASC was extracted with a TaqMan® Fast Cells-to-CT™ kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. RNA extracted from human heart tissue was used as a positive control. RNA was checked on 1% agarose gel.

The cDNA reaction was performed using 0.5–2 μg total RNA with primers from SuperScriptII-kit (Invitrogen, Paisley, UK) according to the manufacturer's instructions. Forward and reverse sequences and expected product size for each specific primer were for α-cardiac actin 5'-ATC TCT GCT GGC CAT GAA AC-3' and 5'-GAT GAG GGA AGG TGG TTT GG-3' (400 bp), for cardiac troponin T 5'-AGA GCG GAA AAG TGG GAA GA-3' and 5'-CTG GTT ATC GTT GAT CCT GT-3' (235 bp), for β-actin 5'-ATC ATG TTT GAG ACC TTC AA-3' and 5'-CAT CTC TTG CTC GAA GTC CA-3' (316 bp), for cardiac β-myosin 5'-CGA GGC AAG CTC ACC TAC AC-3' and 5'-CAT TAA CAG CCT CCA CGG CC-3' (319 bp) and for cardiac myosin light chain 1 (Cmlc1) 5'-GAG GTC GAGTTT GAT GCT TCC-3' and 5'- CGA AGT CCT CAT AGG TGC CTG-3' (300 bp). The PCR reaction was performed with ReddyMix PCR Master Mix (Thermo, Waltham, MA, USA). After the initial denaturation (2 min at 94°C), 35 cycles were performed (20 s at 94°C, 20 s at 53°C, for all set of primers except for β-actin, which was 20 s at 45°C and 1 min at 72°C). The PCR products were run on 1% agarose gel.

Results and discussion

Cell isolation and characterization

After cardiac tissue dispersion, isolated cardiomyocytes showed a typical rod-like morphology (data not shown). hASC obtained following isolation from human lipoaspirates were able to be maintained in culture. Morphologic characterization of the cells showed a typical spindle shape that acquired a homogeneous appearance after 3 weeks in culture (data not shown). Fluorescence-activated cell sorting (FACS) characterization of isolated hASC showed positive expression of surface markers CD105 (100%), CD90 (92.5%) and CD73 (99.6%) and negative expression for both hematopoietic and endothelial cell markers CD45 (0.3%), CD34 (0.2%), CD31 (0.5%) and CD133 (0.1%), as shown previously (8). To test the potential of these stem cells to differentiate along mesenchymal lineages, hASC were treated with conditioned media to induce adipogenic, osteogenic and chondrogenic differentiation. hASC were able to express all three phenotypes when cultured with the appropriate media (Figure 1A).

Cardiomyocyte differentiation

We then tested the potential of hASC to express a cardiomyocyte phenotype following exposure to cell extract isolated from human cardiac atrial tissue. To address the cardiomyogenic potential, we permeabilized the hASC and then treated the cells with and without extract and determined the morphologic changes and expression of cardiac-specific markers after 3 weeks of culture. After exposing cells to the extract, cell viability was estimated to be 15–20%, compared with control non-exposed hASC, which represented a viability of a 100%; 73–80% of the surviving cells expressed cardiomyocyte markers, while the remaining cells (20–27%) retained morphologic characteristics of control hASC.

Phase–contrast microscopy observation of treated hASC (Figure 1B) showed cell-shape changes consisting of an increase in width, the presence of branching fibers and even the presence of binucleated cells. All these features are characteristics of cardiac muscle cells (9).

Immunostaining for cardiac markers revealed expression of cardiac troponin T, Cx43, α-sarcomeric actinin, cardiac troponin I and desmin in extract-exposed but not control hASC (Figure 1C). These results supported the acquisition of a cardiogenic phenotype by the treated hASC. The expression of α-sarcomeric actinin and the inhibitory subunit of the troponin complex, troponin I, cardiomyocyte-specific markers has been proved to be restricted to cardiomyocytes rather than skeletal muscle (9). In addition,

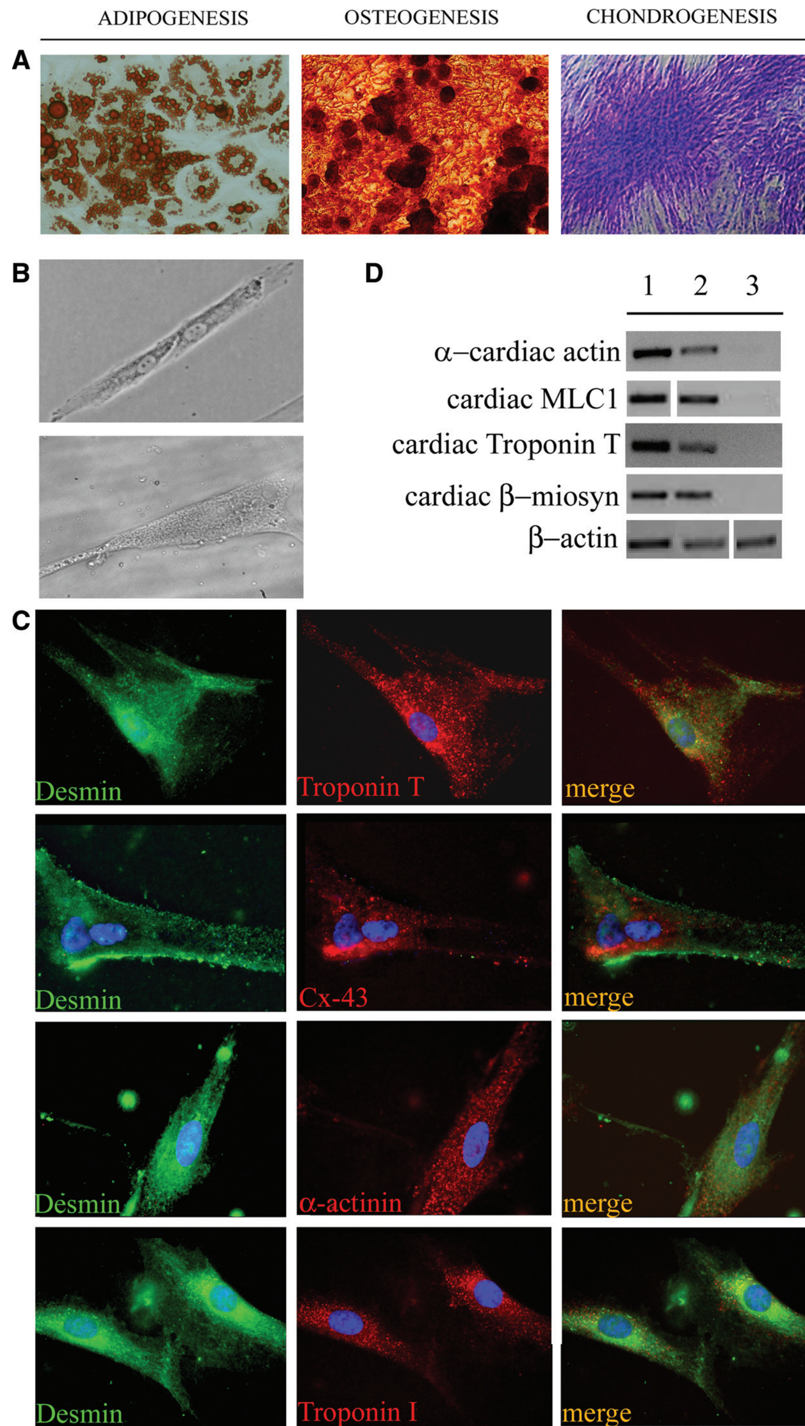

Figure 1. Transdifferentiation of hASC to cardiomyocytes. (A) Adherent hASC were cultured in adipogenic, osteogenic and chondrogenic induction media. Adipogenesis was confirmed by Oil red O staining, osteogenesis by Alizarin red S staining and chondrogenesis by toluidine blue staining. Cells cultured in their respective control medium showed no staining (data not shown). Original magnification ×40. Cardiomyogenic transdifferentiation of hASC after 3 weeks of being exposed to cellular extract obtained from human heart biopsies. (B) Morphologic changes of extract-treated hASC. A cardiomyocyte-like appearance with binucleated and striated cells could be detected. (C) Immunologic detection for cardiac and cell differentiation markers of extract-exposed hASC. Red label for expression of CX43, *α*-actinin, troponin I and troponin T, and green label for expression of desmin. Nuclei stained with DAPI. Original magnification ×63. (D) RT-PCR analysis of expression of cardiomyocyte marker genes. Isolated cardiomyocytes used as a positive control (lane 1) and transdifferentiated cells (lane 2) expressed cardiac *α*-actin (400 bp), cardiac myosin light chain 1 (MLC1) (300 bp), cardiac troponin T (235 bp) and cardiac *β*-myosin (319 bp). No expression of these genes was seen in cells permeabilized but not exposed to extracts (lane 3). Expression of *β*-actin was used as an internal control. Experiments were performed in triplicate, were carried out at least twice and yielded similar results.

we found expression of Cx43, the principal cardiac gap junction protein and a crucial molecule for the conduction of electric impulses as well as for direct intracellular signaling in cardiomyocytes. Cx43 also mediates the expression of an array of genes involved in human cardiomyogenesis (10). Moreover, desmin, which takes part in regulating the differentiation of mesoderm to cardiomyocytes at the beginning of cardiomyogenesis, was expressed (11).

Although previous studies have shown expression of cardiomyocyte markers in spontaneous differentiating populations of hASC, the number of cells found in those experiments was low (0.005–0.07%; mean 0.023 ± 0.03%) and expression was lost when hASC were maintained in culture for 6 weeks (12). In the present study, control hASC were negative for all the cardiomyocyte markers examined (data not shown), suggesting that exposure to the cardiomyocyte extract was necessary to induce expression of the cardiomyogenic markers.

Our immunofluorescence data were confirmed by RT-PCR analysis. After 3 weeks of exposure to the extracts, RT-PCR analysis showed that cardiomyocyte-related genes were expressed in the treated hASC. Figure 1D shows the expression in differentiated cells of cardiac myosin light chain 1 gene, an early marker in cardiomyocyte development, required for sarcomere assembly and essential in cardiomyogenesis for modulating cardiac contractility and cardiomyocyte size and number (13). Moreover, we found expression of late cardiomyocyte development markers, such as α-cardiac actin, cardiac troponin T and cardiac β-myosin, in treated cells (14). On the other hand, control cells showed no expression of any of the cardiac markers tested (Figure 1D).

Data from a number of laboratories have shown that hASC are able to differentiate into cells displaying several features of cardiomyocyte-like cells. These features include the production of cardiac peptides and the expression of multiple structural and contractile proteins. Evidence of cardiomyocyte functionality is only acquired in a time-dependent manner (15). In our study, we were unable to identify any contracting cells during the progress of the experiment.

We show here for the first time that exposure of hASC to a cellular extract obtained from adult human atrial biopsies induces the expression of cardiogenic markers. Although others studies have demonstrated the differentiation of hASC into the cardiomyocyte lineage using cellular extract, these assays were performed with young donors from rodents (7,14,16).

A recent and interesting study (17) did not demonstrate any evidence for the transdifferentiation of either mesenchymal stromal cells or hematopoietic stem cells into functional cardiomyocytes following co-culture with neonatal rat cardiomyocytes. These different observations may be explained either as species differences [Koninckx *et al.* (17) utilized rat cadiomyocytes whereas we used human cardiomyocytes as the starting material], or extracellular factors (secreted from the co-cultured cardiomyocytes) are not sufficient to induce transdifferentiation. In the present study, we utilized cell extracts and permeabilized cells, suggesting activation of the cardiomyocyte program.

Although beyond the scope of the present study, identification of the transcription factors responsible for cardiogenic differentiation will be an important step towards stem cell-based therapy. In this regard, a recent study has shown that the minimal requirement for transdifferentiation of mouse mesoderm to cardiac myocytes is the activation of transcription factors Gata4 and Tbx5 by Baf60c, a Brgl/Brm-associated factor (BAF) chromatin remodeling complex (18). This mechanism could be involved in the present study. Heart extract used to transdifferentiate hASC may potentiate the expression of those factors in the target cells.

In conclusion, the present study provides data that suggest that adult cardiomyocytes retain the capacity to induce cardiomyogenic differentiation of adult human mesenchymal stromal cells. This approach could represent an alternative for inducing cardiomyogenic differentiation that does not rely on gene demethylation or the use of viral vectors.

The relevant finding of this study is the potential use of autologous extracts for induction of stem cell reprogramming. In a therapeutic framework this is essential to avoid immunologic responses from patients.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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