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ORIGINAL RESEARCH

Activated macrophages as a feeder layer for growth of resident cardiac progenitor cells

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Abstract The adult heart contains a population of cardiac progenitor cells (CPCs). Growing and collecting an adequate number of CPCs demands complex culture media containing growth factors. Since activated macrophages secrete many growth factors, we investigated if activated isolated heart cells seeded on a feeder layer of activated peritoneal macrophages (PM) could result in CPCs and if these, in turn, could exert cardioprotection in rats with myocardial infarction (MI). Heart cells of inbred Wistar rats were isolated by collagenase digestion and cultured on PM obtained 72 h after intraperitoneal injection of 12 ml thioglycollate. Cells (1×10^6) exhibiting CPC phenotype (immunohistochemistry) were injected in the periphery of rat MI 10 min after coronary artery occlusion. Control rats received vehicle. Three weeks later, left ventricular (LV) function (echocardiogram) was assessed, animals were euthanized and the hearts removed for histological studies. Five to six days after seeding heart cells on PM, spherical clusters composed of small bright and spherical cells expressing mostly c-Kit and Sca-1 antigens were apparent. After explant, those clusters developed cobblestone-like monolayers that expressed smooth muscle actin and sarcomeric actin and were successfully transferred for more than ten passages. When injected in the MI periphery, many of them survived at 21 days after coronary ligature, improved LV ejection fraction and decreased scar size as compared with control rats. CPC-derived cells with cardiocyte and smooth muscle phenotypes can be successfully grown on a feeder layer of activated syngeneic PM. These cells decreased scar size and improved heart function in rats with MI.

Keywords Cardiac progenitor cells · Macrophage · Myocardial infarction · Rat

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Introduction

Evidence collected over the past several years have shown that the adult mammalian heart contains a population of progenitor cells (cardiac progenitor cells, CPCs) capable of differentiating into cardiomyocytes, endothelial cells and smooth muscle. The putative function of these cells is to maintain normal cardiac homeostasis and repair the heart after myocyte loss (Bearzi et al. 2007; Kajstura et al. 2010).

In rodents these cells have been identified by some stem cell markers, such as c-Kit (CD117, the receptor for stem cell factor), and Sca-1 (stem cell antigen-1) (Oh et al. 2003). In experimental models of myocardial infarction (MI) it has been reported that they may differentiate into cardiomyocytes, endothelial and vascular smooth muscle cells and improve myocardial function (Beltrami et al. 2003).

However, it is difficult to obtain an adequate number of CPCs for transplantation into the heart and several approaches have been applied. One of them is based on the observation that human and mice CPCs can self-organize into three dimensional structures named cardiospheres, that give origin to cardiomyoblasts and endothelial and smooth muscle cells (Messina et al. 2004).

The observation that these cells, isolated from human endomyocardial biopsies, have regenerative potential (Smith et al. 2007) led to efficacy studies in human ischemic heart diseases and heart failure. In fact, the results of a randomized phase I–II clinical trial have been recently reported (Makkar et al. 2012).

Growth and collection of an adequate number of CPC for clinical trials or animal experiments demands at least several weeks, and the use of complex culture media containing growth factors and other components. For example, the cardiosphere method consists of culturing cardiac tissue fragments over fibronectin coated dishes in a cardiac explant medium. After a period of 1–3 weeks a layer of fibroblast-like cells emerge from adherent explants, over which small bright cells migrate. These small cells are collected by gentle enzymatic digestion and seeded in poly-D-lysine-coated wells in cardiosphere-growing medium. 6–10 days later, sphere clusters termed "cardiospheres" appear. Cardiospheres are collected and plated on fibronectin-coated plates where they form a monolayer of cells referred to as cardiosphere-derived cells.

It is known that activated macrophages secrete many growth factors in vitro, such as fibroblast growth factor and insulin like growth factor (Fujiwara and Kobayashi 2005; Hiruma et al. 2012; Oberlin et al. 2009). On this basis, we hypothesized that a feeder layer of activated macrophages could provide the factors needed for growing CPCs.

The possibility that activated macrophages may provide a suitable milieu for growth and differentiation of CPCs is supported by the observation that during the first 2 weeks after an experimental MI, coincident with the presence of a large number of macrophages infiltrating the necrotic zone, there is angiogenesis, arteriogenesis, replication of cardiomyoblasts and entrance into the cell cycle of adult cardiomyocytes in the border zone of the infarct (Vera Janavel et al. 2006), phenomena similar to those reported after injecting cardiosphere-derived CPCs in experimental infarcts (Johnston et al. 2009).

We show herein that culture of isolated rat heart cells on a feeder layer of activated syngeneic peritoneal macrophages (PM) induces the formation of spherical colonies that in successive passages give rise to an almost pure monolayer of cells exhibiting cardiocyte and smooth muscle phenotypes. When injected in rats with coronary occlusion, these cells decreased infarct size and improved heart function.

Fig. 1 **a** Image showing isolated myocytes (*arrows*) seeded on a feeder layer of peritoneal macrophages. **b** After 1–2 weeks of culture over a feeder layers of peritoneal macrophages, spheroidal clusters measuring 50–1,500 μm in diameter were apparent. **c** Isolated cell clusters composed of small brilliant cells, more evident at the periphery. **d** After explant, those clusters developed monolayers with cobblestone-like appearance. **e**–**h** Tissue section of paraffin-embedded cell clusters showing positive cells with *orange* stained cytoplasm after incubation with anti Sca-1 (**e**), anti c-Kit (**f**), anti-smooth muscle actin (**g**) and anti-sarcomeric actin (**h**) antibodies, post treated with peroxidase conjugated avidin and revealed with EAC. Nuclei are stained *blue* with hematoxylin and the *orange* stain of the cell marker is located in the cytoplasm

Materials and methods

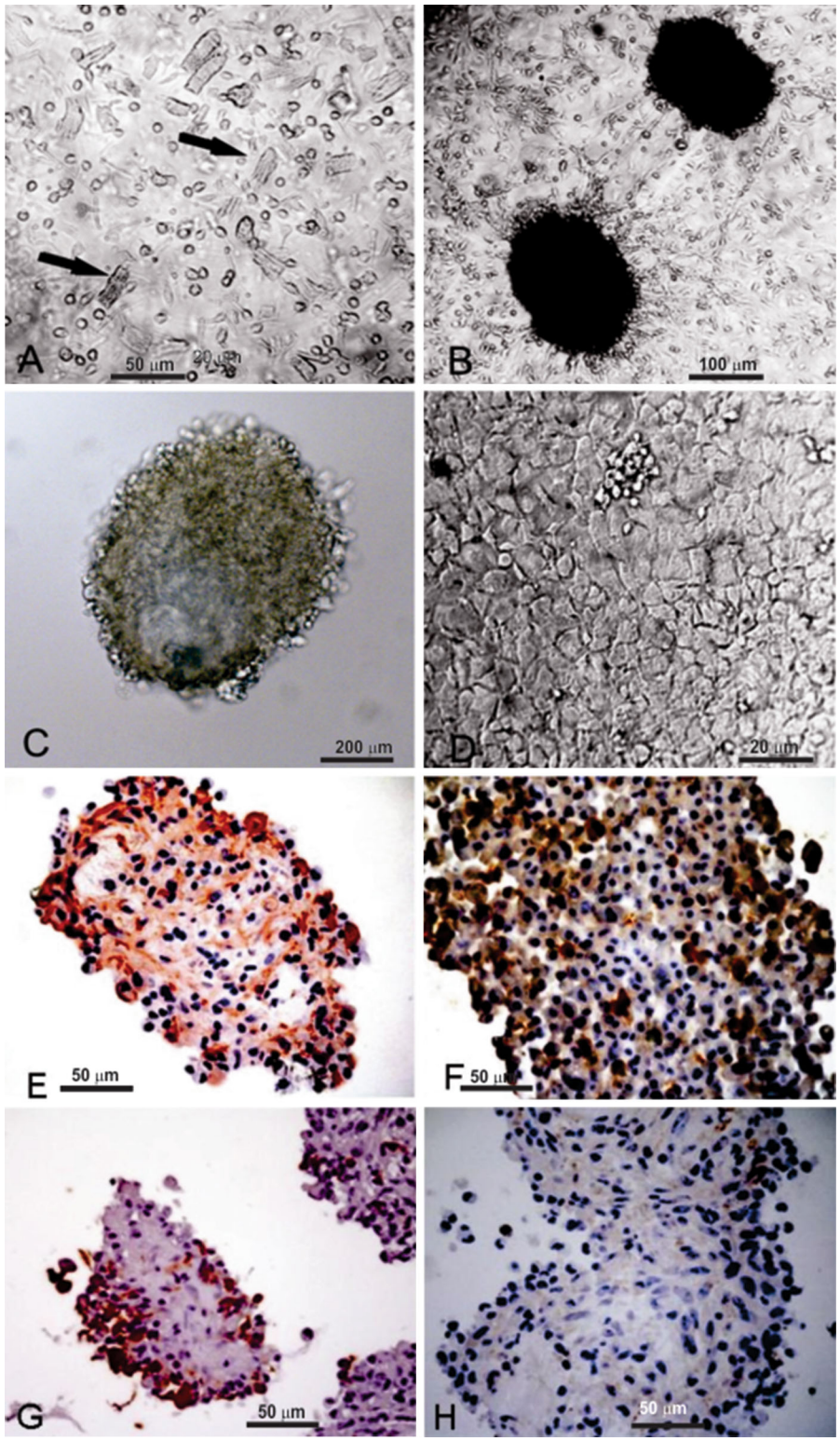
Animals

Inbred 8-week old Wistar-Kyoto rats (School of Veterinary Sciences, La Plata University, Argentina) were used for the experiments.

All procedures were done in accordance with the Guide for Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996) and approved by the Laboratory Animal Care and Use Committee of the Favaloro University, and by the Animal Welfare Committee of La Plata University School of Medicine.

Macrophage collection

PM were obtained according to the technique reported by Delgado et al. (1999). Briefly, rats received an intraperitoneal injection of 12 ml of thioglycollate (Britania, Buenos Aires, Argentina). The peritoneal exudate, collected 72 h later by puncture with a 25-gauge needle,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was diluted in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Invitrogen, Buenos Aires, Argentina) containing 20 % foetal calf serum (FCS) (Internegocios SA, Buenos Aires, Argentina), penicillin (2 %) / streptomycin 2 % (Gibco). Cells were plated in 35 mm diameter culture dishes (Corning, Corning, NY, USA) at 3×10^5 cells/cm^2 for 2 h. After removal of non adherent cells, the medium was replaced and the remaining cells were cultured for 5 days in a 5 % CO_2 atmosphere. The medium was changed every other day. At least 96 % of the adherent cells were macrophages as judged by morphological and phagocytic criteria.

Isolation and culture of CPCs

LV samples of male animals were minced into small pieces and digested with type V collagenase (Sigma-Aldrich, Buenos Aires, Argentina) for 2–3 min at 37 °C under continuous fine tip pipetting. The procedure was repeated until almost no heart fragments were observed (7–8 times). After excluding the coarse tissue fragments by spontaneous sedimentation at 1G the supernatant was centrifuged at 700 RPM, washed twice with PBS and plated for 2 h in 60 mm diameter culture dishes (Corning) with complete medium, to decrease fibroblast contamination. The supernatant was transferred to tissue culture plates containing the adherent PM or to tissue culture plates with no PM feeder layer as control. Figure 1a shows isolated cardiac myocytes seeded on a feeder of PM.

After 24 h the supernatant containing non-adherent cells, including viable and necrotic cardiomyocytes, was discarded. As a result, no cardiocytes were observed in the cultures. Cells were kept in DMEM containing 20 % FCS which was changed every 2 days. After 5–6 days, at 80–90 % of confluence, spherical clusters variable in diameter composed of small bright and spherical cells loosely attached to the tissue culture plate were apparent (Fig. 1b). Following mechanic disruption by continuous pipetting through a fine tip pipette and mild treatment with trypsin–EDTA (Gibco) in order to prevent contamination with adherent macrophages, the clusters were transferred to tissue culture flasks (T75, Corning). After 2–3 passages, cells were collected for the experiments.

Inmunocytochemical studies

The spherical colonies were fixed in 4 % buffered formaldehyde, embedded in paraffin and sectioned at 5 μm. Cell monolayers were fixed in cold methanol or 4 % buffered formaldehyde. Primary antibodies used were anti Flk-1, connexin 43 (Santa Cruz Biotech., Santa Cruz, CA, USA), sarcomeric actin, desmin, c-Kit (Novocastra/Leica, Newcastle upon Tyne, UK), Sca-1 (Bioscience, San Jose, CA, USA), α-smooth muscle actin, vimentin and cytokeratin (Biogenex, San Ramon, CA, USA).

After incubation with the primary antibody, cell monolayers were post-incubated with biotinylated anti-mouse immunoglobulins: (Vector Labs, Peterborough, UK) and post-treated with fluorescein labelled avidin (Vector Labs, Peterborough, UK). After nuclear staining with DAPI, cells were examined with a fluorescence microscope. The paraffin-embedded tissue sections were incubated with the same antibodies but instead of using fluorescein labelled avidin they were incubated with peroxidase conjugated avidin and revealed with EAC (Biogenex). Nuclei were stained with hematoxylin.

Bright field images and fluorescent images were obtained using a Zeiss Axiophot microscope equipped with an Axiocam Zeiss camera.

Cell labeling for location of the injected cells

To track the transplanted cells we used as tracer the fluorescent dye PKH26 (Sigma Aldrich), which mainly binds to the cell membrane. Labeling was performed according to the manufacturer's protocol. Briefly, cells detached by trypsin treatment were washed with serum-free medium, resuspended in dilution buffer from the manufacturer's kit, mixed with a solution containing PKH26 and incubated for 5 min at room temperature. After ending the reaction by adding 2 ml FCS, cells were washed three times with DMEM and observed by fluorescence microscopy. As can be seen in Fig. 3a, b, most cells were labeled.

Surgical procedures and cell implantation

MI was produced in female inbred Wistar-Kyoto rats weighing 220–250 g by left coronary artery occlusion with a 7–0 silk suture under mechanical ventilation. The presence of infarct was verified by pallor at the apex. Ten minutes after ligation, animals ($n = 6$) were injected intramyocardially with 160 μl of phosphate buffered saline (PBS) containing 1×10^6 CPCs, collected from the cobblestone-like cell culture, divided into three equal aliquots in the peri-infarct

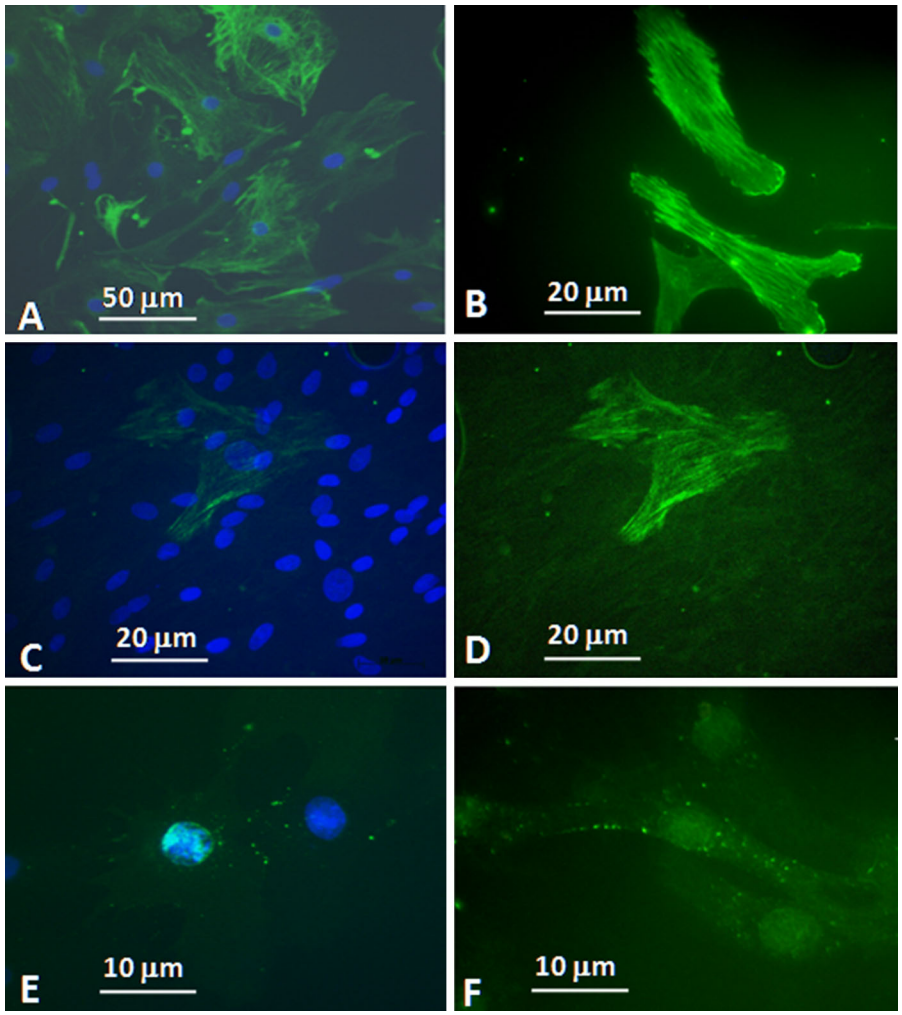


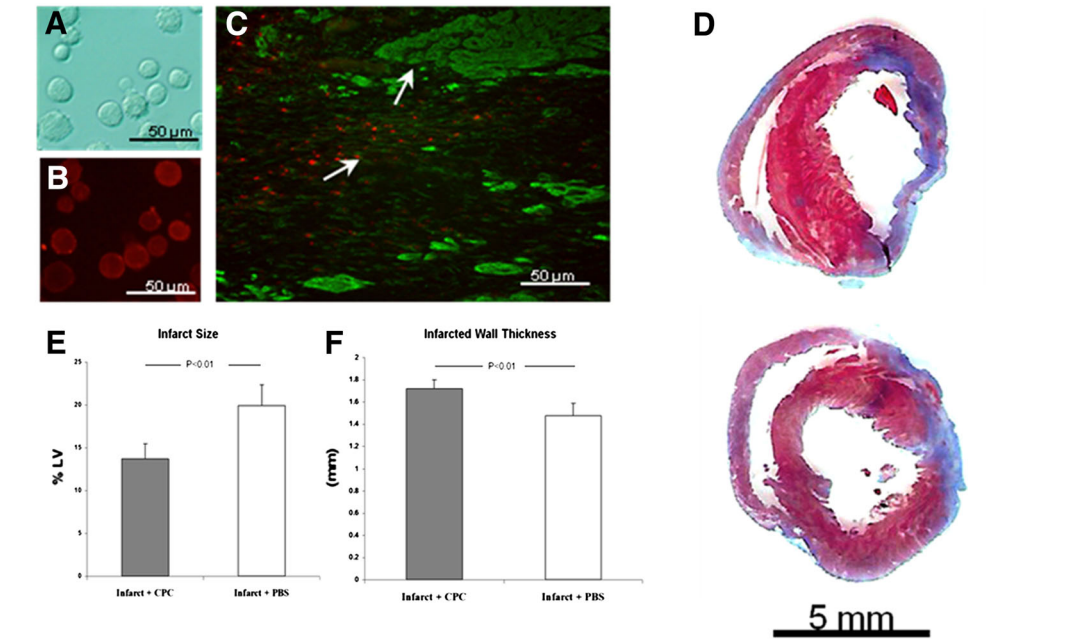
Fig. 2 Immunofluorescent images of the monolayers. Nuclear stain with DAPI. **a** Almost all cells were stained with an anti-desmin antibody. **b** At higher magnification, the longitudinally-oriented smooth muscle actin filaments are apparent. **c** Sarcomeric actin striated sarcomeres. **d** The same cell in **c** with no nuclear stain, showing more clearly the sarcomeric actin filaments. **e** Almost all cells in the monolayer show in their surfaces numerous punctated areas intensely stained after incubation with an anti-connexin 43 antibody. **f** Higher magnification of connexin 43-positive structures

zone. Control animals (n = 6) were similarly injected with 160 µl PBS. Four additional animals received the same amount of CPCs labeled with PKH26. Six sham-operated rats underwent thoracotomy and heart exposure but no coronary artery ligation. Three weeks later, an echocardiogram was performed. Immediately after the procedure, the animals were euthanized and the hearts removed for histological studies.

Histological studies

The hearts were immersed in 10 % buffered formaldehyde for 48 h and sectioned transversally to the main axis in three slices, one at 1 mm from the atrioventricular sulcus, one at 1 mm from the apex, and one mid way between the apex and the atrioventricular sulcus. Slices were embedded in paraffin. Tissue sections measuring 3 µm in thickness were stained with hematoxylin-eosin and trichrome. Scar size and infarcted wall thickness were determined on Masson's trichrome stained sections with a morphometry analysis system (Image ProPlus 4.5, Media Cybernetics, Rockville, MD, USA).

The hearts of the rats injected with the PHK26 labeled cells were sectioned at the middle of the LV, and snap frozen in liquid nitrogen. Five micrometer sections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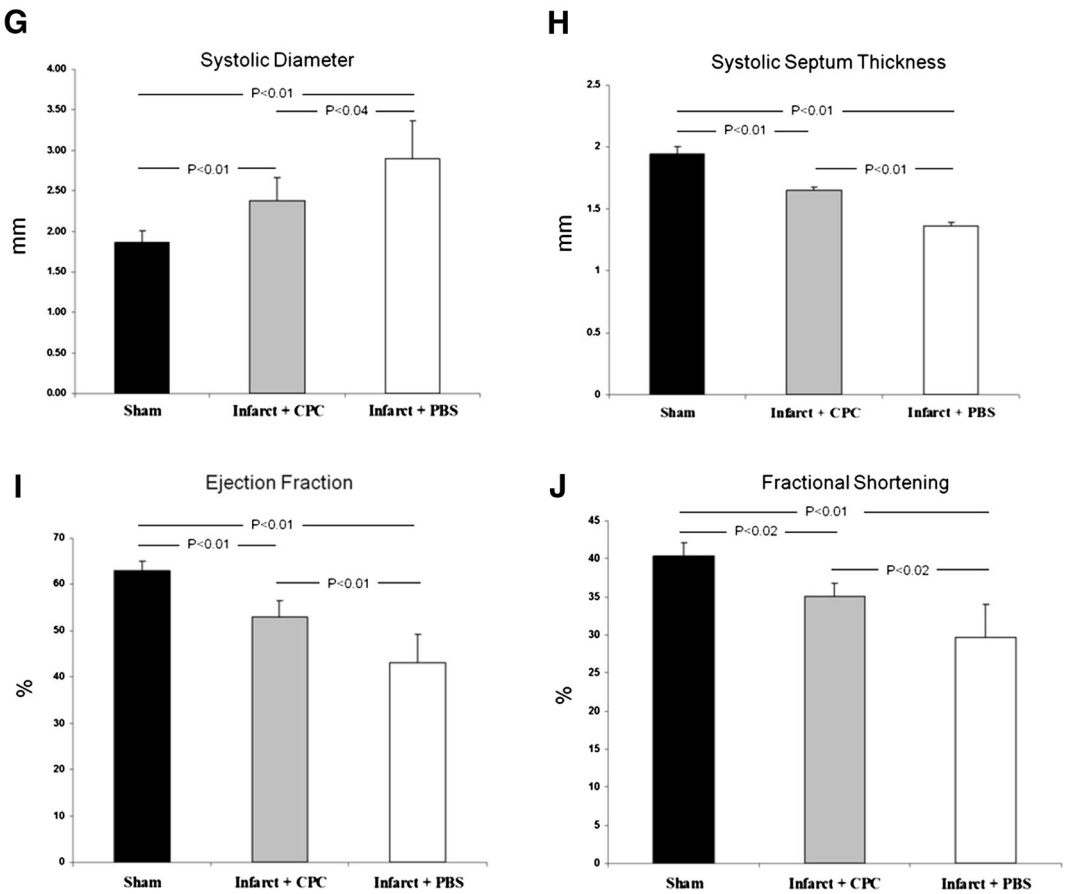
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◀**Fig. 3** **a** and **b** Cell labeling for location of the injected cells: under Nomarski optics, all cells appear stained with PKH26. **c** At 21 days after implantation, many labeled cells derived from the monolayers, with sarcomeric actin-positive cytoplasm (*small arrow*), are present in the interstitium and in the neighborhood of heart muscle cells (*large arrow*) (Masson's trichrome stain). **d** Transversal sections of hearts from a PBS-treated mouse (*upper*) and a CPC-treated mouse (*lower*) at 21 days after ligature of the left coronary artery: the difference in the size of the infarcted area (*blue*) between injected with CPCs and PBS is apparent. **e** Infarct size was significantly smaller in mice injected with CPCs. **f** Infarcted wall thickness was significantly higher in CPC-treated mice. **g–j** Left ventricular function: at 3 weeks after treatment, CPC-treated mice displayed lower systolic diameter (**g**), higher systolic wall thickness (**h**), higher ejection fraction (**i**) and higher fractional shortening (**j**) than mice injected with PBS

obtained with a cryostat were counterstained with DAPI and examined with a fluorescence microscope.

Echocardiographic studies

Under superficial anesthesia, transthoracic 2D guided M-mode echocardiography was performed. Measurements were averaged from five consecutive cycles. From a short axis at the level of the papillary muscles, LV end-diastolic dimension (EDD) and volume (EDV), and end-systolic dimension (ESD) and volume (ESV), interventricular septum thickness at end systole (SST), and end diastole (SDT), anterior wall thickness (AWT), fractional shortening (FS) and ejection fraction (EF), calculated as $EF = 100 \times (LVEDV - LVESV)/LVEDV$, were recorded.

Statistical analysis

Continuous variables are reported as mean ± SD. Differences between continuous variables were analyzed with one-way ANOVA followed by Scheffé's test. A p value <0.05 (2-sided) was considered significant. Statistical analysis was performed with SPSS®11.0 software (SPSS Inc, Chicago, IL, USA).

Results

Cardiac progenitor cells isolation, culture and phenotypic characterization

One to two weeks-old cultures of isolated LV cells showed spheroidal clusters composed of small brilliant round cells measuring 50–1,500 μm in diameter on the feeder layer of activated PM (Fig. 1b, c). They were loosely attached and could be collected mechanically by pipetting or by mild trypsin incubation. At that time no cardiomyocytes were present in the plates, on account that they had been removed during the previous washings. In control plates seeded with the heart suspension but without PM feeder layer, no adherent cells were observed at 1 week after culture, and only a few spherical isolated cardiocytes were floating in the culture medium.

Culture of the cell clusters in uncoated plasticware with DMEM containing 20 % FCS produced monolayers of cells with cobblestone-like shape that were successfully maintained for more than ten passages (Fig. 1d).

The immunohistochemical study of paraffin embedded spherical colonies revealed that they were composed of a cell population expressing stem cell markers (Sca-1 and c-Kit) and scarce cells with smooth muscle and myocyte markers (Fig. 1e–h). Labelled cells were distributed at random mostly in the periphery of the cell colonies, and apparently there were no differences between small and large colonies.

Almost all cells in the monolayers displayed desmin filaments in their cytoplasm (Fig. 2a). For each culture, a variable proportion of cells expressed smooth muscle actin (Fig. 2b) or the cardiac marker sarcomeric actin, with many of them showing clear-cut sarcomeres (Fig. 2c, d). In addition, most cells expressed connexin-43 in their surface (Fig. 2e, f). The search of cytokeratin, a cytoplasmic marker for mesothelial cells, and of Flk-1 for endothelial cells, was consistently negative. Beating cells were never observed.

Fate of injected CPCs

As can be seen in Fig. 3c, at 21 days after injection many cells labeled with PKH26 were present in the peri-infarct area. Some of the labeled cells displayed cytoplasmic sarcomeric actin, but were smaller than the neighboring native cardiocytes.

Changes in infarct size and LV morphometry

At 3 weeks after MI, morphometric analysis of explanted hearts from control animals showed increased size of the collagen scar and infarct wall thinning as compared with animals receiving syngeneic myoblasts (Fig. 3d–f).

LV function

Cardiac function results are shown in Fig. 3g–f. At 3 weeks after MI, the group receiving CPCs showed, as compared with the PBS group, higher FS (35.63 ± 1.81 vs. 29.72 ± 4.27 %, $p < 0.05$), EF (53 ± 3.5 vs. 43 ± 6.3 %, $p < 0.01$) and SST (1.65 ± 0.03 vs. 1.36 ± 0.03 mm, $p < 0.01$). On the other hand, LVESD was lower in CPCs than in PBS animals (2.38 ± 0.28 vs. 2.89 ± 0.47 mm, $p < 0.01$). LVEDD and AWT showed no significant differences among the groups.

Discussion

The discovery in the adult heart of a reservoir of progenitor cells, able to renew themselves and differentiate into multilineage phenotypes (Beltrami et al. 2003) has provided a new approach for cardiac cell therapy after MI (Barile et al. 2007).

The expression of specific cell surface markers, such as c-Kit or Sca-1, permitted their isolation by cell sorting and amplification by posterior growth in specific culture media. Transplantation of Sca-1 derived cells in experimental infarction induces angiogenesis and neomyogenesis with improvements in cardiac function (Tang et al. 2007; Wang et al. 2006) and intramyocardial injection of c-Kit derived cells in the infarct border zone induces cardiac regeneration including muscle and vessel neoformation (Hosoda 2012).

Recently, these cells have been employed in a clinical trial of patients with heart failure of ischemic etiology undergoing coronary artery bypass surgery. The results showed improvement in both global and regional LV function, infarct size reduction and increased viable tissue (Bolli et al. 2011).

CPCs in amounts adequate for experiments and clinical trials can also be obtained by the cardiosphere method. Messina et al. (2004) and Smith et al. (2007) demonstrated that small cardiac explants cultured on poly-D-lysine induce the appearance of self-assembling spherical clusters termed cardiospheres, composed of undifferentiated and cardiac-committed cells. When they are re-plated and expanded in monolayer cultures, a relevant number of cardiosphere-derived cells are obtained. When injected into experimental infarcts, these cells engraft in the injured heart, differentiate, secrete pro-angiogenic/cardiogenic cytokines and confer functional benefits.

A recent phase I–II clinical study of intracoronary cardiosphere-derived cells in patients with recent acute MI and LV dysfunction showed promising results (Makkar et al. 2012).

On account that in experimental MI intracardiac stem cells, especially those derived from cardiospheres, provide the greatest functional benefit when compared with extracardiac stem cells (Li et al. 2012), developing new approaches for culturing intracardiac stem cells is of interest.

Our results show that culturing isolated rat heart cells on a feeder layer of syngeneic PM induces the appearance of cell clusters resembling the cardiospheres described by Messina et al. (2004). Their expansion in monolayers produced cells expressing differentiation markers of cardiocyte and smooth muscle lineages such as smooth muscle actin, desmin, sarcomeric actin and connexin 43. In addition, they decreased the scar size and improved function in experimental MI. Apparently, many cells survived 21 days after injection, as shown by the labeling experiments.

Despite some differences, all reported techniques used to obtain CPCs demand complex differentiation media containing several growth factors, such as basic fibroblast growth factor, leukemia inhibitory factor, epidermal growth factor, cardiotropin-1, thrombin and synthetic media for growth of Sca-1 cells or for cardiospheres (Smith et al. 2007; Shenje et al. 2008; Andersen et al. 2009; Li et al. 2009; Ling et al. 2013).

Apparently, the feeder layer of activated macrophages made unnecessary the incorporation of those products to the culture medium. Although it is known that activated macrophages, especially those belonging to the M2 subpopulation, secrete cytokines and growth factors engaged in angiogenesis and tissue repair (Fujiwara and Kobayashi 2005; Oberlin et al. 2009; Hiruma et al. 2012), the factors that may induce the formation of CPCs and cells with cardiac phenotypes were not investigated in the present work. It is known that macrophages, derived either from peritoneum or from circulating monocytes only proliferate in culture when provided with growth factors (van der Zeist et al. 1978; Genovesi et al. 1989). Since in our experiments the macrophage culture was not irradiated, we could not discard the possibility that the incorporation and posterior growth of heart-derived cells could provide growth factors able to induce

macrophage proliferation. However, the fact that the cell clusters and monolayers contained cells with stem cell and smooth and heart muscle markers, which are not expressed by macrophages, makes that possibility unlikely.

However, once the cell clusters were re-plated in uncoated tissue culture flasks, only conventional tissue culture media and FCS were required for culture expansion.

The cell clusters obtained in the primary culture of isolated heart cells on syngeneic macrophages showed some phenotypic characteristics similar to the cardiospheres described by Messina et al. (2004) such as expression of the stem cell markers c-Kit and Sca-1. In addition, some cells showed smooth muscle actin and sarcomeric actin markers, but in contrast with the expanded cardiosphere-derived progenitors, neither cells with endothelial markers nor fully differentiated cardiocytes or beating cells were observed.

In summary, the present study shows that a suspension of isolated heart cells grown on a feeder layer of syngeneic activated PM yields cells belonging to the cardiac and smooth muscle lineages, and that these cells, in turn, can decrease scar size and improve myocardial function in rats with MI. The approach used permits obtaining CPCs in adequate amount for experiments at low cost and reasonable simplicity, suggesting that it could be possibly employed for isolation and differentiation of stem cells of other tissues and organs.

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Conflict of interest The authors declare that they not have conflict of interest.

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