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Localization and origin of cardiac CD117-positive cells: identification of a population of epicardially-derived cells in adult human heart

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Summary

During heart morphogenesis, epicardial cells undergo epithelial-mesenchymal transition giving origin to a population of epicardially derived cells that play a crucial role in the development of most cardiac cell lineages. Considering the hypothesis that epithelial-mesenchymal transition of epicardial mesothelium can generate cardiac primitive cells in the adult heart, we have examined *in vivo* and *in vitro* the epicardium and subepicardium of normal human adult hearts and of pathological hearts from patients with chronic ischemic heart failure for the presence of CD117-positive cells with epithelial and mesenchymal markers expression. The number of CD117-positive cells increased significantly in the subepicardium of pathological hearts and sloped down towards myocardium, remaining still elevated with respect to normal hearts. While cells with typical epithelial proteins expression formed an intact layer on the surface of the normal hearts, CD117-positive cells were localized mainly in the subepicardium and expressed mesenchymal markers in the pathological hearts. Epithelial-mesenchymal transition, induced *in vitro* by several growth factors known to accumulate in the ischemic myocardium, gave origin to epicardially-derived cells with CD117 expression. These data support the hypothesis of epicardial origin of cardiac primitive cells in the adult human heart.

Key words

Cardiac stem cells; epicardium; epithelial-mesenchymal transition; epicardially-derived cells; cardiac regeneration; ischemic cardiomyopathy

Introduction

During heart morphogenesis, epicardial cells undergo an epithelial-mesenchymal transition (EMT) generating a population of epicardially derived cells (EPDCs). These cells populate the subepicardium and subsequently invade the myocardium, where they play a crucial role in myocardial compaction and in the development of the coronary vasculature, fibrous skeleton of the heart, cardiomyocytes (Zhou et al. 2008), and Purkinje fibres (Dettman et al. 1998; Muñoz-Chápuli et al. 2001). The process of EMT is characterized by the loss, or translocation to different cell compartments, of epithelial markers such as POD-1 (epicardin), Bves, cytokeratin, β-catenin and E-cad-

herin, and by the acquisition of mesenchymal markers such as M-cadherin, vimentin and Tie-2 (Zeisberg et al. 2009), upon the activation of signalling regulated by several growth factors, among which TGFβ, HGF, EGF, PDGF, FGF, and Snail/Slug transcription factors (Thiery et al. 2006). Recently, undifferentiated cells able to give rise to the cells of cardiac lineages have been observed also in the adult human heart, raising questions concerning their origin and biology. Moreover, complex networks that orchestrate EMT have been found to be activated in the process of cardiovascular progenitors formation (Martínez-Estrada et al. 2010). Considering the hypothesis that EMT of epicardial mesothelium can generate cardiac primitive cells in the adult heart, we examined epicardium and subepicardium of normal human adult hearts and of pathological hearts from patients with chronic ischemic heart failure for the presence of CD117-positive cells with epithelial and mesenchymal markers expression. Moreover, the purpose of the present study was to investigate whether EMT takes place also in the adult human heart and contributes to cardiac tissue regeneration in chronic pathological conditions giving origin to CD117-positive cells. To this aim, and due to the lack of feasibility of *in vivo* tracing studies of adult human cardiac stem cells, we have adopted an integrated approach based on the analysis of adult cardiac tissue sections in normal and pathological conditions and on the study of human epicardial cell biology *in vitro*.

Materials and Methods

Cardiac tissue samples

Samples of atrial appendages and left ventricle from normal hearts were obtained from donors died for reasons other than cardiovascular disease (n = 11, mean age 41 ± 12 years, 7 males, 4 females). Pathological heart samples were derived from patients with ischemic cardiomyopathy (n= 20, mean age 55 ± 5.5 years, 14 males, 6 females, mean ejection fraction 25 ± 1%, without previous heart surgery), at the time of heart transplantation. Specimens were collected in conformity with the Declaration of Helsinki.

Epicardial cell culture

The epicardium was stripped down from the auricles, placed on plastic Petri dishes covered with cardiac fibroblast-derived matrix and cultured in 60:40 (v/v) MCDB (US Biological, Swampscott, MA, USA) and DMEM-low glucose (Invitrogen, Paisley, UK) supplemented with 2% fetal bovine serum (Invitrogen), 9.4 ng/ml linoleic acid, 1 mg/ml bovine serum albumin, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite, 0.1 mM ascorbic acid-2-phosphate, 1 nM dexamethasone, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (all from Sigma-Aldrich, St Louis, MO, USA), in order to obtain the outgrowth of epicardial cells *in vitro*. For EMT induction, 3 nM transforming growth factor-beta1 (TGFβ1), platelet-derived growth factor-BB (PDGF-BB), fibroblast growth factor-2 (FGF2), hepatocyte growth factor (HGF) or epithelial growth factor (EGF, all from PeproTech, London, UK) were added to culture medium.

Tissue and cell staining

Cardiac tissue samples were fixed in 10% neutral buffered formalin and embedded in paraffin. Cells attached to culture dishes were fixed in 4% paraformaldehyde. Primary monoclonal antibodies against CD117 (MAB1163, Millipore), α-sarcomeric actin, vimentin (both from Sigma-Aldrich), M-cadherin (Millipore, Temecula, CA, USA), b-catenin, or polyclonal goat anti-human BVES, cytokeratin 5/6, POD-1 (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used. Immunohistochemistry was performed with indirect immunoperoxidase technique employing the IHC Select Immunoperoxidase Secondary Detection System (DET-HP1000, Millipore). For the immunofluorescence studies, sections were incubated with primary antibody followed by secondary antibody conjugated with fluorescein or rhodamine (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Nuclei were counterstained with DAPI. Microscopic analysis was performed with a Leica DMLB microscope equipped for epifluorescence (Leica Microsystems, Wetzlar, Germany).

Statistical analysis

All numerical data are presented as mean ± SD of at least three separate experiments. Statistical differences were evaluated with Student's two-tailed *t* test for comparison between two groups; ANOVA with post hoc Bonferroni's *t* test was used when more than two groups were compared. $p < 0.05$ was considered statistically significant.

Results

Cardiac primitive cells become more numerous in the subepicardium of hearts with ischemic cardiomyopathy

The presence of CD117-positive cells was analyzed by immunofluorescence (fig. 1A, B). In the normal hearts, there were 409.6±11.6 cells/100 mm^2 in the left atrium and 248.0±67.3 cells/100mm^2 in the left ventricle. In the hearts with chronic ischemic cardiomyopathy CD117-positive cells were significantly more numerous than in the normal hearts: the left atrium contained 3573.0±874.9 and the left ventricle 3243.0±1134.0 cells/100 mm^2 (fig. 1C).

Both in the normal and in the pathological hearts, CD117-positive cells were more numerous within the epicardium than in the myocardium. In the normal hearts, the number of cells in the epicardium was 27.5-fold and 19.5-fold higher than in the myocardium in the left atrium and left ventricle, respectively. In the hearts with ischemic cardiomyopathy, the epicardium within the left atrium contained 38-fold more cells than the myocardium, while in the left ventricle the number of CD117-positive cells in the epicardium was 42-fold higher with respect to the myocardium.

Subepicardial cells express epithelial and mesenchymal markers in the hearts with ischemic cardiomyopathy

We characterized by immunohistochemistry and immunofluorescence the CD117-positive cells present in the epicardium/subepicardium of human adult normal and

pathological hearts with ischemic cardiomyopathy. Intriguingly, only the normal adult human hearts were covered with mesothelium, which, by contrast, was missing from the surface lining of the pathological hearts. On the surface of the normal hearts, all cells expressed POD-1 (epicardin), Bves and cytokeratin 5/6, while in the pathological hearts cells positive for these epithelial markers were scattered across the subepicardium (fig. 2). Moreover, mesenchymal markers, such as M-cadherin and vimentin, were present in the subepicardial cells in the pathological hearts.

Mesothelial cells of adult epicardium undergo EMT

Our model of adult human epicardial cell culture in the presence of subepicardial fibroblasts-derived extracellular matrix allowed us to obtain the outgrowth of epithelial sheet with polygonal cells and typical epithelial markers expression (e.g. β-catenin localized at the cell membrane). In order to examine whether the epicardial cells from adult human heart can undergo EMT, several cytokines known to be potent inducers of EMT in different cell lines were added to the culture medium. In the presence of TGFβ, HGF and EGF, signs of EMT were observed since the intercellular contacts were lost (β-catenin translocated from the cell membrane into the nucleus) and the cells acquired a spindle-like shape and presented motile properties (fig. 3). The addition of PDGF-BB and FGF2 alone did not induce EMT. As demonstrated by immunofluorescence, 32±1,8% (n=4) of EPDCs expressed a stem cell factor receptor, CD117.

Discussion

Although the localization and the fate of CD117-positive cardiac primitive cells in the adult heart have been already extensively studied (Beltrami et al. 2003, Limana et al. 2005), we have demonstrated that the number of CD117-positive cells increased significantly in the pathological hearts with ischemic cardiomyopathy and that the gradient of these cells sloped down from epicardium towards myocardium. These cells expressed epithelial and mesenchymal markers that include cytoplasmic or membrane proteins and transcription factors implicated in the process of EMT or in its regulation. Successively, the induction of EMT in the epicardial cells of adult human heart *in vitro* provided the connection between these cells and cardiac primitive CD117-positive cells, known to be able to give rise to cardiomyocytes, smooth muscle and endothelial cells. From the above observations it follows that EMT of mesothelium is a likely way of cardiovascular progenitor cell generation in the adult human heart.

To the best of our knowledge, this is the first study in which the process of EMT has been successfully induced and observed in the adult human epicardial cells *in vitro*. In a previous study, Wada et al (2003) were able to induce EMT in rat mesothelioma cells; however, both the requirements for transformed cell culture and the neoplastic cell fate can not be compared with those of adult human normal epicardial cells. A recent study (van Tuyn et al. 2007) addressed the differentiation properties of EPDCs, however it failed to demonstrate EMT, since the epicardial-derived cells with mesenchymal characteristics were generated following epicardium cell trypsin-

ization *in vitro*. Similarly, the protocol established by Smart et al. (2009) permits to obtain the outgrowth of EPDCs, but not mesothelial epicardial cells, from myocardial tissue fragments.

As is the case with most tissues and organs with self-renewing capacity, the preservation of stem cells pool requires specific microenvironment that protects the slow-cycling, undifferentiated, and self-renewing stem cells. We have recently described the changes in the subepicardial compartment of cardiac wall in the course of ischemic cardiomyopathy, regarding both CD117-positive cells number and extracellular matrix components (Castaldo et al. 2008), which favour cell survival, proliferation and migration. However, the activation of cardiac stem cells in pathological conditions may result in their progressive differentiation or functional deterioration, leading to the exhaustion of functionally competent cardiac stem cells, as suggested by our recent observations of epicardium and subepicardium in the adult human normal heart and in pathological hearts explanted because of chronic ischemic heart failure (Di Meglio et al. 2009).

The most common modality of heart ischemia is associated most commonly with subendocardial infarction, and more rarely with full thickness wall necrosis, while the epicardium and the subepicardial region typically retain a relatively better blood supply. Hence, the possible epicardial origin and subepicardial localization of cardiac primitive cells, described in our study, seem advantageous to the survival of cells and to the stimulation of their proliferation and differentiation in pathological conditions.

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Figures

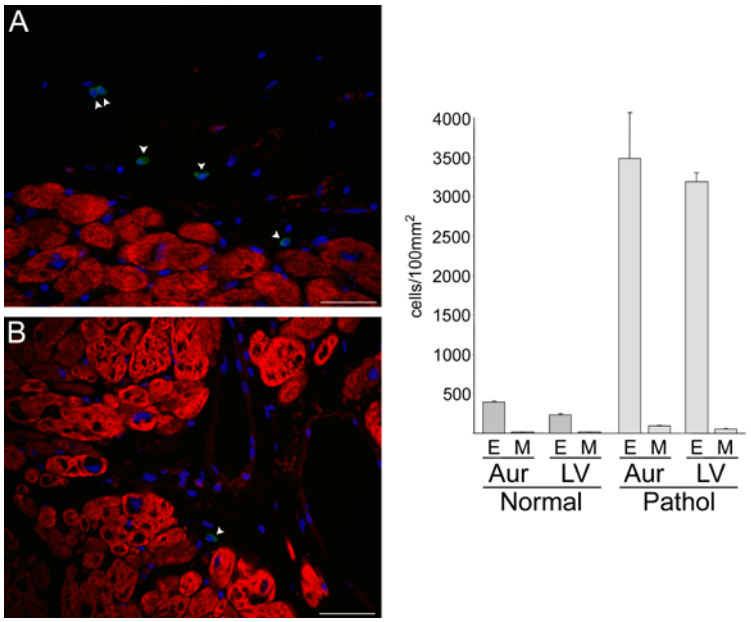

Fig. 1 – Localization and number of CD117-positive cells in the adult human heart. Representative photomicrographs of cardiac primitive cells (CD117, green; α-sarcomeric actin, red; nuclei, blue) in the epicardium of normal hearts (A) and in the myocardium of pathological hearts with ischemic cardiomyopathy (B); scale bar corresponds to 50 μm. With respect to normal hearts, the number of CD117-positive cells increased 13,3-fold in the subepicardium and 4,7-fold in the myocardium of the atrium of hearts with ischemic cardiomyopathy (Pathol), while in the left ventricle the increase reached 8,8-fold and 6,4-fold, respectively (C). E, epicardium; M, myocardium; Aur, auricle; M, myocardium.

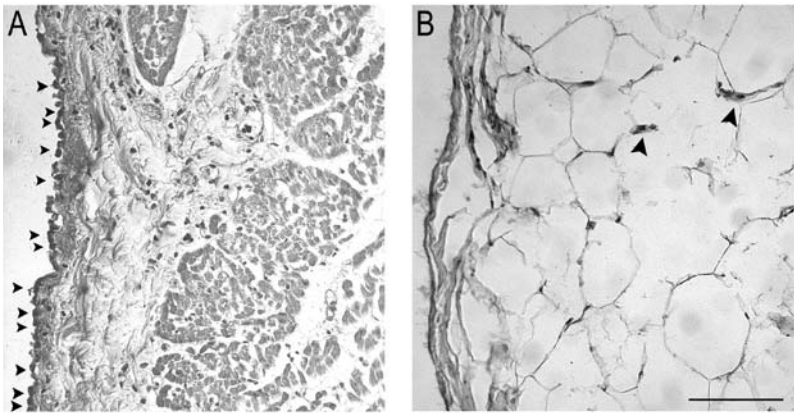

Fig. 2 – Epithelial markers expression in epicardial cells. In normal hearts (A), POD-1 positive cells, revealed by immunohistochemistry, were confined to the mesothelial layer of epicardium, whereas in the pathological hearts (B) these cells were scattered through subepicardial connective tissue. Scale bar corresponds to 100 μm.

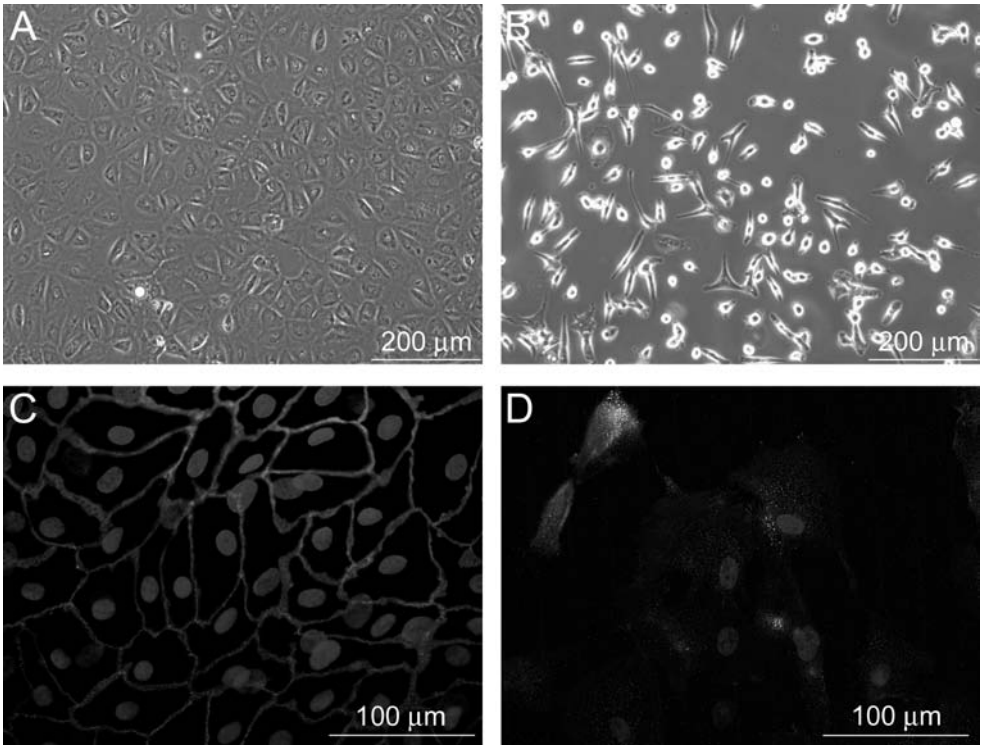

Fig. 3 – EMT of epicardial cells from adult human heart *in vitro*. Epicardial cells formed a monolayer of polygonal cells (A) with β-catenin confined to cell membrane at intercellular junctions (C). In the presence of specific growth factors, cells became scattered and spindle-shaped (B), while β-catenin translocated in the cytoplasm and nucleus (D). A and B: phase contrast; C and D: immunofluorescence for β-catenin, nuclei were counterstained with DAPI.