

Dormant cardiac stem cells: A promising tool in cardiac regeneration

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Abstract. Stem cells represent an old niche with various new potential therapeutics. Besides drug treatment, reperfusion procedures and surgical revascularization, stem cell therapy could be a good option in ischemic cardiac diseases. A study was performed on a small group of cases who died of cardiac arrhythmia secondary to scarring myocardial infarctions. Tissue cardiac samples were taken from these cases (from the anterior and lateral wall of the left ventricle), for microscopy examination, in order to investigate the presence of cardiac stem cells (CSC). Multiple series of histological sections were also performed and examined, along with immunohistochemical analysis (IHC). The cells were identified in close contact with the residual ischemic cardiomyocytes, in the proximity of the myocardial collagenous scar, in old myocardial infarctions. They were activated by hypoxic ischemia and were influenced by the capillary microvascular density and the interstitial micro-environment conditions. In chronic

intermittent ischemia they seem to turn themselves from dormant quiescent cells into activated progenitor committed cells.

Introduction

Cardiac ischemic disease and myocardial infarction are disabling conditions that result in the appearance of scar fibrosis and loss of myocardial contractile function. The chronic intermittent cardiac ischemia following this event is one of the main causes of death among human population (1-3).

Until recent times, cardiac ischemic diseases were treated only by drug therapy, associated with surgical revascularization procedures and with reperfusion treatments (4,5). In the last decade, modern modalities of treatment of ischemic myocardium, involving gene therapy, stimulation of interstitial stem cells and heart transplant, have also been investigated (6).

In this situation, a new dynamic view considers that cell death and cell restoration in the heart are a part of organ homeostasis, although the rate of myocyte renewal/turnover is very low (7,8).

In the heart regeneration process, important information could be obtained by detection and characterization of a subpopulation of cardiac progenitor cells (CPC) and cardiac stem cells (CSC), which are immature, but already committed stem cells. These heterogenic group of cells are concentrated in specific areas of the heart, such as the atria or pericardium (8) and express mesenchymal stem cell markers, such

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as c-kit, CD34, CD90 and CD105 and sometimes extracellular markers, such as Rex1, Nanog and Sox-2 (9,10).

Apart from this, CSCs have also been identified based on the expression of early cardiogenesis markers such as platelet-derived growth factor receptor- α , and fetal liver kinase-1 (11).

Patients and methods

Case selection for human tissue specimens. From a study group of 30 cases with various ischemic heart diseases, in an interval of one year, a series of cases consisting of 3 males (aged 69, 75 and 82 years) and 2 females (aged 58 and 88 years) were selected for histopathology, in order to determine the presence of CSC.

They all died of cardiac arrhythmia secondary to scarring myocardial infarctions (~4-6 weeks). The lesions were located subendocardial and intramural.

The old myocardial infarctions were associated with variable myocardium fibrosis, subsequent to a long standing ischemic cardiopathy and atherosclerosis. The study was performed according to the World Medical Association Declaration of Helsinki and the tissue specimens were collected according to national legislation, using a protocol approved by the local Bioethics Committee of 'Sf. Pantelimon' Emergency Clinical Hospital (Bucharest, Romania). All patients provided a signed informed consent regarding hospitalization, treatment and the possible future publication of data.

Tissue sampling and stains. Tissue samples from the heart were taken for microscopy investigation. The fragments were harvested from the anterior and lateral wall of the left ventricle.

The selected tissue samples were fixed in 10% neutral buffered formalin (pH 7.0) for 24-48 h and paraffin-embedded. Sections were cut at 5 μm and stained with standard H&E and van Gieson.

Tissue samples have been divided into appropriate-sized slices for conventional microscopy and immunohistochemistry. Multiple series of histological sections were also performed and examined.

Semi-thin sections (~1 μm) were stained with Toluidine blue and examined under light microscopy, in order to determine morphometric analysis in the interstitial area.

Immunohistochemistry. Immunohistochemical analysis (IHC) was done using sections displayed on slides treated first with poly-L-lysine. IHC was performed on 3 μm thick sections from formalin-fixed paraffin-embedded specimens.

The method used was an indirect triserial avidin-biotin-complex technique, with a NovoLink Polymer detection system which utilizes a novel control polymerization technology to prepare polymeric HRP-linker antibody conjugates, according to the manufacturer's instructions (Novocastra).

Briefly, the procedure comprised: deparaffination in toluene and rehydration in alcohol series, washing in phosphate-buffered saline (PBS), blocking with normal serum, 5-min incubation with primary antibody 60-min incubation with post-primary block for 30 min, then with NovoLink Polymer for 30 min. Sections are further incubated with the substrate/chromogen 3,3'-DAB and counterstained with Meyers' hematoxylin.

The antibodies used for IHC were: CD56/N-CAM (clone: 1 B6, RTU, Novocastra), CD117/c-kit (T595, RTU, Novocastra) and CD34 (QBend/10, RTU, Novocastra).

Antigen retrieval techniques (thermal or enzymatic pre-treatment) for the aforementioned antibodies were done, according to the producer's specifications.

Negative control was made by using a primary irrelevant antibody or by replacing the secondary antibody with PBS. Positive control was made comparatively with the expression of antibody investigated in specific cells or tissue structures (positive internal control on slides).

To ensure the reliability of the experimental study, internal quality control of histopathologic and IHC techniques were performed as part of an implemented and certified quality assurance system (ISO 9001/2018).

All slides were examined and photographed on an AccuScope Imager microscope. Digital images acquired with an incorporated software program were processed and analyzed with Microsoft Office Picture Manager, running under Windows 10.

IHC assessment and statistics. The distribution of marker-positivity was assessed using the modified Quick score method (12), which takes into account the intensity and distribution of the IHC reaction: 0, negative (no staining); 1, weak (only visible at high magnification); 2, moderate (readily visible at low magnification); 3, strong (strikingly positive at low magnification).

Statistical analysis was carried out for the obtained data, using the Student's t-test, along with descriptive statistics for mean, median and standard error (SE). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

We identified the cells in the proximity of the myocardial collagenous scar, in close contact with the residual ischemic cardiomyocytes. CSCs were located peri-fibrillar and interstitial, adjacent to the plasma membrane (sarcolemma) of the cardiac muscle fibers.

The distribution along the muscular fibers, showed that they were oriented parallel or perpendicular to the longitudinal axis of the cardiomyocytes.

From the morphological point of view, CSC are small, plump and ovoid, mononuclear cells, with well defined borders, scarce cytoplasm and centrally located nuclei, sometimes hardly noticeable (depending upon the incidence of the cut section) and with a high nuclear to cytoplasm volume ratio.

The morphometry analysis showed that these cells, measured at x400 magnification, had a minimum diameter of 6 μm and a maximum diameter of 16 μm , with a mean of 11 μm and SE, $\pm 3 \mu\text{m}$.

CSC stained positive for CD117 (Fig. 1), but stained negative for CD56 and CD34. Positive intern control was used for correct IHC assessment in mast cells for CD117, in neural fibers for CD56 and in capillary vessels for CD34. CD56 stained positive in the gap junctions of the cardiomyocytes (Fig. 2).

The microvascular density in the adjacent area of the myocardium infarction, assessed by CD34 revealed a high density capillary network, with activated CSC (Fig. 3 inset).

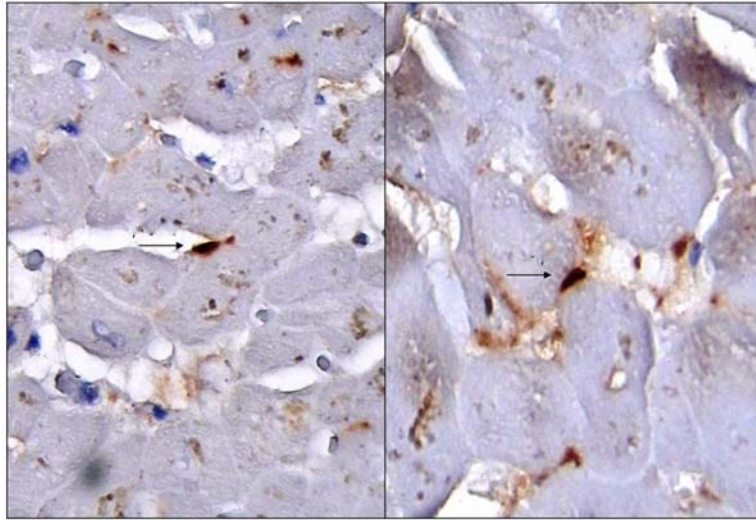


Figure 1. Positive staining of interstitial CSCs for CD117, in close contact with residual cardiomyocytes, IHC, x400 (black arrows). IHC, immunohistochemical analysis; CSCs, cardiac stem cells.

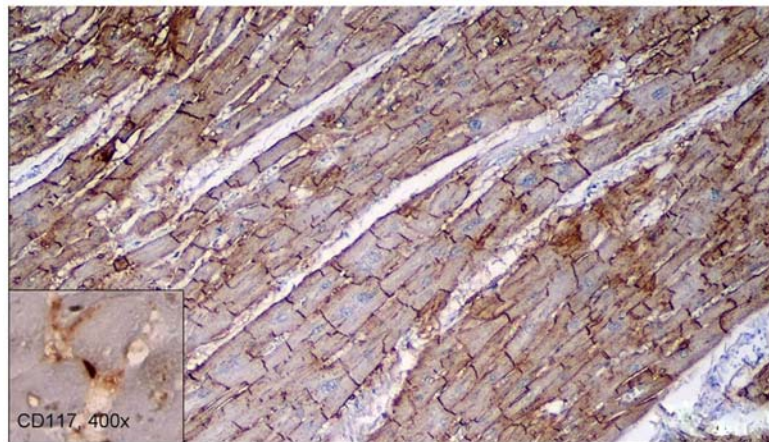


Figure 2. Positive staining of CD56 in the gap junctions in residual ischemic cardiomyocytes; IHC, x100 (inset, CD117-positive activated CSC; IHC, x400). IHC, immunohistochemical analysis; CSCs, cardiac stem cells.

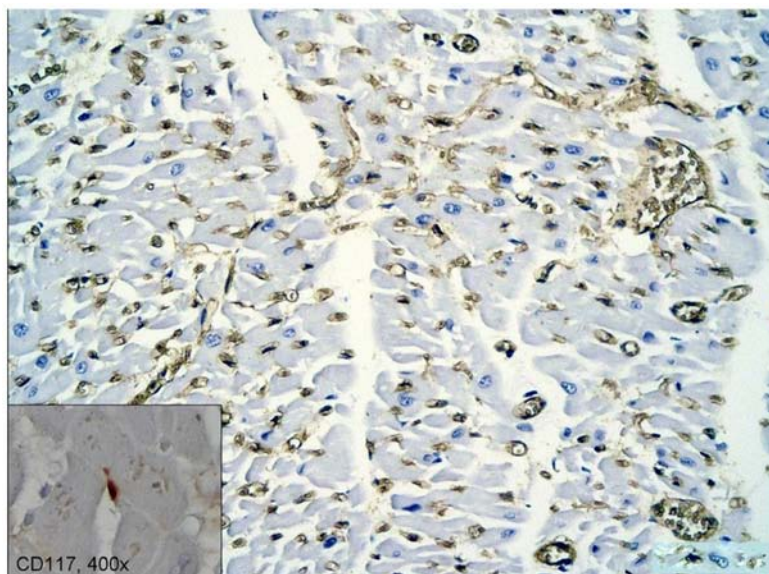


Figure 3. High micro-vascular density of capillary network assessed by CD34 in the interstitium of residual myocardium, IHC, x200 (inset, CD117-positive activated CSC, IHC, x400). IHC, immunohistochemical analysis; CSCs, cardiac stem cells.

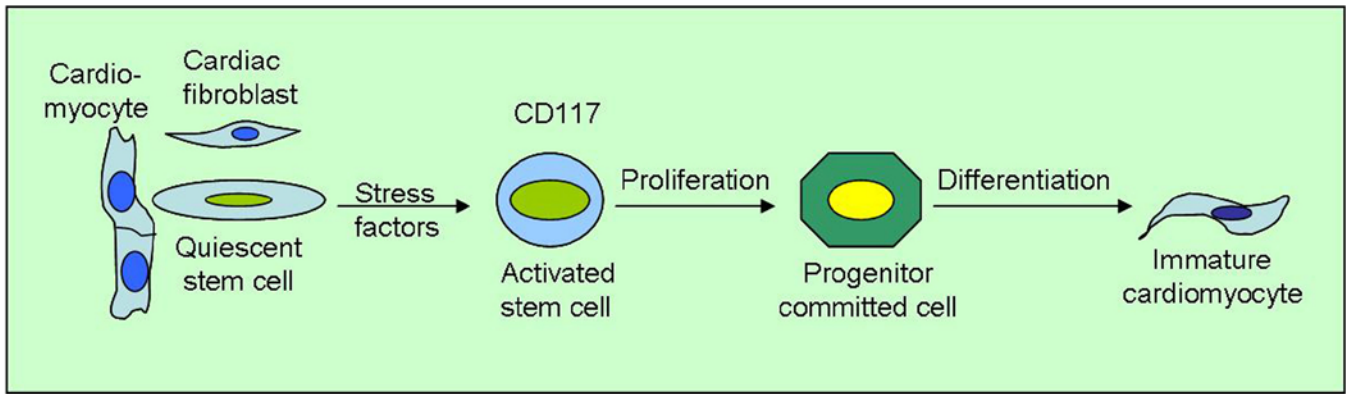


Figure 4. Schematic diagram illustrating the transformation sequence of the dormant (quiescent) stem cell in stress conditions of the surrounding microenvironment.

The size and shape of these cells are influenced by the vascularization of the surrounding micro-environment and possibly by other stromal cells.

Discussion

Various new potential therapeutics have been shown in stem cells. Stem cells have been discovered in various tissues: including in small bowel (intestinal stem cells), in the skin (epidermal stem cells), skeletal muscle (satellite stem cells) and liver (oval cells). The small bowel mucosa is an example of a rapidly self-renewing tissue. Its origin is represented by dedicated stem cells, located at the crypt base (13). Intestinal stem cells provide by differentiation mature functional cells. They follow a migratory path from the base of the crypt toward the villi. In skeletal muscle they were identified up to 17 days in humans and up to 14 days in animal experimental models (mice) post-mortem (14) and they were also demonstrated in human cadavers up to six days after the estimated time of death (15). Stem cells exist not only in skeletal muscle, but also in the myocardium.

Activated CSC are pluripotent and are involved in regeneration of the myocardium. In normal myocardium they are quiescent and adopt a dormant state (retaining the regenerative capacity), but they become activated in stress conditions, particularly in chronic ischemia. They grow through symmetrical cell division, being influenced also by the nearby stromal cells.

It has been shown (16) that they are in close contact with telocytes, which may play a role in their activation. According to the study, CSC and telocytes form a 'tandem', both morphological and functional, advancing the idea that this binary unit might be useful in cell transplantation procedures.

In the animal kingdom, heart can be seen as a self-renewing organ, in some metazoans. For instance, in zebrafish, within 2 months after a significant heart injury, it can be assisted to cardiac regeneration, facilitated by proliferation and subsequent dedifferentiation of cardiomyocytes (17,18).

In humans, the neonatal heart is associated with considerable growth and cellular proliferation of cardiomyocytes. The heart of newborn continues to grow and proliferate in their first week of life. The post-natal period is marked by increased

apoptosis, active cardiac remodelling and modest cellular turn-over (19).

In adults, regarding the cardiomyocytes, the only response to stress is hypertrophy, secondary to an increased workload or death, after acute ischemic injury. In the second situation, the necrotic cardiomyocytes decay and are slowly removed by macrophages (which phagocytose the cellular debris) and is later replaced by collagenous tissue with scar formation. In long standing ischemia after a devastating event such myocardium infarction, CSC from cardiac niches are activated.

The quiescent CSC become active stem cells, which proliferate, turning into committed progenitor cells and finally giving rise to immature cardio-myocytes and new vascular structures (Fig. 4). The process, however, is slow and requires a long time for renewal *in vivo* (20).

Cardiac committed progenitor cells obtained from adult hearts adhere in culture to form a three-dimensional spherical structure named a cardio-sphere. These cardio-spheres (up to 150 microns in size) have an enormous proliferative capacity, generating one million cardio-spheres in a period of one month. They represent a heterogeneous cell population, consisting of an outer layer of proliferating cells (c-kit-positive) and an inner layer of differentiated, contractile cardiomyocytes (desmin-positive) (21,22).

CSC possess growth factor-receptor systems that after activation regenerate the infarcted myocardium, improving ventricular function and long-term survival (23).

CSC originating from the infarction area had a higher proliferative potential and a greater propensity to migrate in comparison to the cells originated from a healthy myocardial area. Also, the expression level of several specific markers of cardiogenic differentiation was higher in the cells from the infarction area than in cells from the healthy myocardium (24).

In vitro, CSC have self-renewal capacities and *in vivo* they are able to differentiate into three cell types: cardiomyocytes, endothelial cells and vascular smooth muscle cells. It is thought that the cardiac stem/progenitor cells express a variety of markers, the most known and used being CD117/c-kit, CD34, integrin β -1 (CD29), Islet-1, SCF (stem cell factor also known as c-kit Ligand or steel factor). CD117 and CD34 are also

markers for telocytes (Cajal cells) from intestinal epithelium and other tissues (25).

Stem cell factor provides proliferation, differentiation and survival of stem cells. It assists the recovery of cardiac function after myocardial infarction by increasing the number of cardiomyocytes and vascular channels.

In addition, *in vivo* lineage tracing studies suggest that another population of CPC expressing TBX18 and WT1 reside in the epicardium (26).

Furthermore, freshly isolated heart specimens taken by myocardial endo-biopsy could add significant and valuable information into understanding of myocardial biology of development, injury, and aging with a great impact on therapy (27).

In conclusion, we consider that chronic intermittent myocardial ischemia activates the intrinsic regenerative potential of dormant CSC, these being influenced by the capillary microvascular density and the interstitial micro-environment conditions. In these conditions, the cells seem to turn themselves from dormant quiescent cells into activated progenitor committed cells.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZC and MCE performed the histological examinations and IHC, and had major contributions in the writing of the manuscript. BS, DP and VDC analyzed and interpreted the patient data. MCo, MCTD, NB and CC performed the literature research and contributed to the writing of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was conducted according to the World Medical Association Declaration of Helsinki and the protocol used was approved by the local Bioethics Committee of 'Sf. Pantelimon' Emergency Clinical Hospital (Bucharest, Romania). All patients provided a signed informed consent regarding hospitalization, treatment and the possible future publication of data.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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