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PhD THESIS

STEM CELL THERAPY FOR MYOCARDIAL INFARCTION: MECHANISMS AND MOLECULAR SIGNALS BY WHICH TRANSPLANATED CELLS CONFERS CARDIOPROTECTION IN ISCHEMIA

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ABSTRACT

Cardiovascular diseases are the leading cause of death worldwide and one of the most important clinical manifestations of these is represented by ischemic heart disease. This pathological condition of the heart is characterized by reduced coronary flow in a certain region of the heart, which results in the appearance of myocardial necrosis due to ischemia. The development of heart failure after myocardial infarction is strongly related to alterations in the geometry, function and structure of heart, that are collectively called "ventricular remodeling".

The current treatment of heart failure can only improve symptoms but can not reverse the loss of myocardial tissue by replacing dead cells with new contracting cardiomyocytes (CMC). The only current alternative to the more severe pathological manifestations of heart failure is heart transplantation, but this therapeutic intervention is limited, not only because of the high costs that are involved, but also due to unavailability of donors.

In these circumstances, stem cell therapy has brought hope in cardiology for the treatment of bouth myocardial infarction and heart failure, being motivated by encouraging pre-clinical results obtained in the last 10 years. These experimental results led to the initiation of the first clinical trials, but their effect has proven far more than modest, showing an improvement in left ventricular ejection fraction of only 2-4%. Moreover, some trials have shown that there are no significant differences in the function of the myocardium with and without cell transplantation, in the long term. The reason was that transplanted cells are poorly retained in infarcted heart and that most of them died after transplantation. Thus, the hypothesis was advanced that the improvement is not due to myocardial regeneration, but is produced by the stem cell-secreted biomolecules, which can help for a period of time the remodelling process. These findings have led to reconsideration of previous observations in the field of stem cells and have called for the development of new strategies for improving survival, grafting and cell differentiation after transplantation.

The main types of stem cells considered as options for regenerative medicine are embryonic stem cells (ESC) and adult stem cells (ASC). ESC are generated from the inner cell mass of the blastocyst before implantation. They are pluripotent cells, being able to differentiate into derivatives of all three germ layers: ectoderm, endoderm and mesoderm. ESC can be maintained in undifferentiated state by culture in specific conditions. When these conditions are changed,

cells begin to differentiate and thus they can be manipulated to be committed to cardiomyocytes (CMC). However, CMC generated from ESC have an immature phenotype, making them currently not usable in clinical settings.

ASC are defined as stem cells/progenitors that remained in the body after the development of the organism. Among these, mesenchymal stem cells (MSC) are multipotent cells isolated from adult bone marrow and other tissues, which may give rise to adipocytes, osteoblasts and condrocytes and, with lower efficiency, to neurons, CMCs, pancreatic beta cells or liver cells. Endothelial progenitor cells (EPC) are another type of ASC. EPC are hematopoietic cells isolated from bone marrow or peripheral circulation. Although apparently they do not differentiate in CMC, EPC helps myocardial regeneration by promoting angiogenesis and providing protective paracrine signals to CMC.

The present work is divided into two major sections. The first part briefly describes the current state of knowledge and is organized in 3 parts: (i) Cellular and molecular mechanisms involved in cardiac ischemia-reperfusion; (ii) “Heart conditioning” – therapeutic strategy for cardiac ischemia-reperfusion injury; and (iii) Stem cells and their therapeutic potential in ischemic heart disease. The second part of the thesis ("Original Contributions") shows the contribution of this work to the enrichment of the knowledge in the stem cell field and is organized into eight chapters. These chapters presents the standardization and improvement of the experimental models used (the generation of new cell lines of embryonic and adult stem cells and experimental models of myocardial ischemia-reperfusion in vivo, ex vivo and in vitro), and the results obtained on these models; results published (or under publication) in international journals in the field.

A constant concern throughout the entire PhD period was to increase the efficiency of stem cell differentiation into contractile CMC and to take advantage for their properties to obtain a structural and functional improving of the infarcted myocardium. Therefore, in the first part, the studies have focused on the derivation of ESC lines, and getting a good yield of their differentiation into CMC. The results showed that in vitro differentiation in contractile cardiac cells of ESC derived from RAP mice (for the first time reported in the literature) is dependent on the initial number of stem cells used and their level of compaction in embryoid bodies (EB). Replacement of foetal bovine serum (which is most commonly used for in vitro differentiation of

ESC) with KO-SR (Knock-Out Serum Replacement) during aggregation of ESC in EB resulted in improved differentiation in CMC by increasing the percentage of contractile EB generated.

With regard to studies carried out on adult stem cells, they focused mainly on the analysis of the capacity of MSC to induce myocardial regeneration after ischemia-reperfusion injury. For these studies were standardized several experimental models of ischemia-reperfusion, namely: the experimental mouse models of *in vivo* ischemia (mouse with myocardial ischemia-reperfusion obtained by transient ligation of the left coronary artery and the mouse with hindlimb ischemia obtained by permanent ligation of femoral artery), the experimental model of *ex vivo* cardiac ischemia-reperfusion using the Langerdorff system and models of *in vitro* ischemia-reperfusion by incubating heart (foetal, neonatal and adult) sections in conditions of ischemia (the presence of an ischemic buffer that chemically mimics the ischemia and atmosphere of 1% O_2). The main original findings obtained on these models are:

1. Both foetal and adult myocardium are affected by ischemia, as evidenced both by decreased tissue viability after ischemia, and by low levels of phosphorylated enzyme ERK1/2. Although foetal myocardium responds to ischemia by general manifestations of ischemic tissue (increased levels of SDF and VEGF secreted into the culture medium), it is more resistant to post-ischemic reperfusion, as demonstrated by returning of viability to baseline after one day of reperfusion. Besides, foetal myocardium does not respond to the ischemia through the formation of scar tissue, TGF-β levels being decreased in ischemia-reperfusion. Unlike foetal myocardium, adult myocardium responds to ischemia by decreasing levels of CGL and scar tissue formation (shown by increased levels of TGF-β at both mRNA level and in the supernatant). Reperfusion induced inflammatory response (increased IL-6 after 1 hour post-ischemic reperfusion), as well as increased levels of HGF and c-Met, involved in repair process.

2. Both under normoxia, and hypoxia, MSC and EPC have cardioprotective properties and stimulate angiogenesis, through which they can contribute to the regeneration of the ischemic myocardium. None of these populations, alone, can sustain an effective angiogenesis, translated by chemotaxis, adhesion and proliferation of endothelial cells (EC) in vitro. MSC are able to promote migration and adhesion of EC, but cannot sustain their proliferation after adhesion. Unlike MSC, EPC shows complementary effects on angiogenesis, being able to stimulate EC proliferation, but not their adherence to the substrate. Thus, the combination of factors secreted by MSC and EPC has a strong positive effect on the behaviour of EC in vitro, indicating the

possibility of effective therapies for vascular regeneration through simultaneous use of several populations of stem/ progenitor cells.

3. Subcutaneous transplantation of MSC protects the myocardium from ischemia-reperfusion injury, indicating a possible therapeutic strategy for multiple clinical applications. Transplantation of MSC in a remote region from the myocardium ("remote transplantation"), by a non-invasive method, proliferates but did not migrate detectably from the injection site. These cells produce and secrete a variety of molecules with systemic paracrine role of which were identified PTX3 and TSG-6 (proteins with cardioprotective effects), and a number of cytokines pro- (TNF-α, IL−1β) and anti-inflammatory (IL-10, IL-1Rn), involved in modulating the inflammatory process. The hearts isolated from mice transplanted with MSC shows a significantly reduced infarct area and improved cardiac function after ischemia-reperfusion, compared with hearts isolated from control mice.

4. As another original result, the mouse model of cardiac ischemia-reperfusion emphasizes the importance of ECG recording during LCA ligation, to validate both the ligature, and reperfusion of the vessel. Without adding any major disadvantage in terms of procedure, ECG recording allows for objective validation of ligation (by showing ST height elevation and prolongation of QTc segment) and reperfusion, after releasing the ligation of the vessel (by returning the ECG to normal profile), thus replacing the subjectivity given by the evaluation of the reddish color in the ischemic region of the heart. Moreover, ECG provides important information about the severity of myocardial changes caused by ischemia, demonstrating a correlation between electrocardiography and histological changes obtained after ischemia-reperfusion.

The results presented in this thesis describe some important aspects of molecular signals and mechanisms involved both in the pathophysiology of ischemia-reperfusion injury and stem cell transplant for myocardial infarction, thus contributing to the progress of basic research in the field of both stem cells and cardiovascular disease.