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PhD THESIS

FACTORS MODULATING THE THERAPEUTIC POTENTIAL OF ENDOTHELIAL PROGENITOR CELLS FOR NEOVASCULARIZATION OF ISCHEMIC TISSUES

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KEYWORDS

Neovascularization
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Chemotaxis
Cardioprotection

THESIS ABSTRACT

In the current context in which cardiovascular disease is the leading cause of mortality, elucidating the mechanisms underlying the process of new blood vessels formation in the adult (neovascularization), intensively preoccupies scientific community. Although numerous experimental studies and clinical trials using stem cells / progenitors to induce angiogenesis and myogenesis showed encouraging results (Gersh et al., 2009, Heil and Schaper, 2004; Lipinski et al., 2007, Mitsos et al. 2012), the mechanisms by which individual populations of stem cells / progenitor help improve cardiac function post-infarction are still incompletely understood (Gersh et al., 2009). A better understanding of these cellular therapies could increase their efficiency. Equally important, considering the ischemic environment which stem cells face after

transplantation into the infarcted myocardium, understanding the paracrine properties of stem cells in hypoxic conditions is essential. This would allow devise therapeutic strategies personalized to each patient, to overcome possible obstacles caused by pathological peculiarities. Optimization strategy could be done by choosing the most appropriate cell populations or combinations of the population, the use of only the factors secreted by them, by the choice of route of administration, and timing in relation to acute vascular accident. Furthermore, the cells to be injected may be preconditioned by stimulating with various cytokines or by blocking or over-expression of cytokine receptors (Herrmann et al., 2011, Richardson et al., 2013).

In this context, this thesis highlights several stem/progenitor cell populations with angio-/vasculogen potential and some of the mechanisms by which they contribute to neovascularization of ischemic tissues. Additionally to beneficial effect of these cell types on neovascularization, aiming to improve the function of infarcted myocardium, their ability to protect cardiac myocytes from hypoxic stress has also been evaluated. The thesis also investigates contribution of various paracrine factors to the therapeutic potential of stem/progenitor cells and modulation of their expression or of the paracrine properties of cells by hypoxia. In the last part of the thesis the influence of SDF-1/CXCR4 axis on the paracrine role of the progenitors and in cardiac remodeling after myocardial infarction (MI) are assessed.

The thesis is organized into two main parts containing 143 figures, of which 105 figures in the second part, 7 tables (in the second part), and 290 citations and extends over 233 pages.

In the first part, the Current State of Knowledge, organized into 6 chapters, firstly describes available therapeutic options, highlighting the need to develop new therapeutic strategies (Chapter I.1.). Then, after a brief description of the structure of blood vessels (Chapter I.2.), the physiological mechanisms of neovascularization are explained into detail (Chapter I.3.). Initially the terms vascular-, angio- and arteriogenesis are defined and then stages of angiogenesis (reorganization of basement membrane, of extracellular matrix, and cytoskeleton during endothelial cell (EC) migration, vessel maturation and stabilization) are described. In the fourth chapter (Chapter I.4.) several models that can be used to experimentally study vessel formation are presented, while the penultimate chapter (Chapter I.5.) describes key factors modulating neovascularization. The last chapter (Chapter I.6.), dedicated to the description of various strategies that may be used to induce neovascularization, firstly passes over the main types of

stem cells and their general properties, and then the characteristics of the two types of cells involved in neovascularization: endothelial progenitor cells (EPC) and mesenchymal stem cells (MSC) are given in detail. Finally a synthesis of current state of experimental and clinical studies is presented, successes and limitations being described.

In the second part of the thesis, the Original Contributions, studies were organized into six chapters. Initial studies focused on identifying potential populations of endothelial stem cells/progenitors (Chapter II.1.). The results show that from adult and fetal tissue sources that had been used (peripheral blood, umbilical cord blood, Wharton's jelly and bone marrow) the two types of EPC described in the literature (early and late) and mesenchymal stem/progenitor cells can be obtained. Endothelial potential of the latter is limited, but their secretory phenotype suggested their paracrine potential to stimulate neovascularization.

Numerous studies indicate that early and late EPC contribute to neovascularization by separate mechanisms, the early EPC having paracrine role, while late EPCs, being highly proliferative, are able to incorporate in new vessels (Asahara et al., 1997; Hur et al., 2004 Sieveking et al., 2008). It is also recognized that the formation of new blood vessels can be modulated by using a combination of angiogenic factors, the process being magnified when these factors are administrated/released sequentially (Freeman and Cohen, 2009, Hao et al., 2007). On the other hand, a study that examined the effect of co-administration of early and late EPC on neovascularization, using an in vivo model of hind limb ischemia, showed that the two cell types have a synergistic effect (Yoon et al., 2005). These data raise questions whether the beneficial effect obtained is dependent on factors released by early EPC, as a consequence of a dynamic response of these cells to the conditions encountered *in vivo*. To answer this question, using two *in vivo* models, we sought whether factors secreted *in vitro* by early EPC may improve neovascularization induced by late EPC (Chapter II.2.). The results show that in the presence of factors secreted *in vitro* by early EPC, late EPC included in a Matrigel plug, transplanted subcutaneously in mice, can organize into vascular structures merging with the vascular network of the host organism. Also late EPC transplantation in ischemic muscle, in the presence of factors secreted by early EPC, significantly increased tissue perfusion, an effect that could not be obtained at a similar level when late EPC or early EPC secreted factors were administered separately. Thus, we can conclude that factors released *in vitro* by early EPC are able to

significantly stimulate late EPC organization into new blood vessels, transplantation of early EPC being not mandatory.

Identification of factors contained by conditioned medium, that are responsible for its beneficial effect, will enable designing of a therapeutic compound that could be produced on a large scale and may be administered to an ischemic site, together with the late EPC, in order to improve tissue perfusion. Thus, from the results of the *in vivo* studies, we sought further to describe the paracrine factors responsible for the beneficial effect of early EPC (Chapter II.3.). We also investigated the influence of hypoxia on the secretory properties of early EPC and angiogenic and cardioprotective capacity of factors released by early EPC under normoxia and hypoxia. The cytokine profiling of early EPC revealed that they secrete numerous pro-angiogenic factors (VEGF, SDF-1, PlGF, Ang2, ET-1, IL-8, MCP-1, MMP10), and anti-angiogenic factors (TIMP-1, CXCL4), being thus able to contribute to maintaining of an angiogenic balance that would prevent pathological angiogenesis. We also showed that although hypoxia modulates secretory profile of early EPC, inducing an overall increase in secretion of factors involved in angiogenesis, including VEGF, these changes do not significantly influence the angiogenic effect of condition medium. Thus, to note, factors released by early EPC, both in normal conditions and in conditions of oxygen deprivation, are able to induce EC proliferation and migration, EC organization in vascular cords and protect cardiac myocytes from hypoxia induced apoptosis. Contribution of VEGF to paracrine angiogenic properties of early EPC was also investigated. The results showed that the presence of VEGF in conditioned medium from early EPC is not essential for the migration of EC or for EC organization in cords on Matrigel.

Next paracrine properties of mezenchimal stem cells (MSC) were investigated, following their ability to induce EC adherence and proliferation and to protect cardiomyocytes from hypoxia-induced apoptosis (Chapter II.4.). Also, their potential was compared to early EPC properties and was evaluated under hypoxic conditions. The results showed that the factors secreted by MSC stimulate EC chemotaxis and adherence, but they are not able to induce the proliferation of EC; they also have beneficial effects on ischemic cardiomyocytes. Secretory activity of MSC is slightly modified as a result of exposure to hypoxia, but these changes do not affect their paracrine properties. Effects induced *in vitro* by early EPC are complementary to those induced by MSC, stimulating proliferation, but not EC adhesion. Both of these effects can be induced

simultaneously by combining the conditioned medium, suggesting that factors released from each of the two cell types are not sufficient to complete angiogenesis. This suggests that the combination of these two cell populations or of the factors secreted by them, angiogenesis can be induced successfully.

Neovasculogenesis is a process dependent on the activity of various cytokines such as VEGF, bFGF, angiopoietins and SDF-1 (Parsons-Wingerter et al., 2000 Visconti et al., 2002). The mechanisms by which they orchestrate neovascularization are still disputed. It is known that a gradient of SDF-1 is formed at an ischemic site, which causes the mobilization of bone marrow cells expressing CXCR4 and the receptor-ligand interaction triggers signaling cascades involved in the growth, proliferation and cell survival (Ganju et al. 1998, Petit et al., 2007, Roland et al., 2003). In this context, we sought further to evaluate the expression profile of SDF-1 by early EPC and we investigated whether paracrine role of these cells in neovasculogenesis is mediated by SDF-1 (Chapter II.5.). Also, given that SDF-1/CXCR4 interaction is exploited increasingly more to stem cell therapy after MI (Zhang et al., 2007), we proposed to study the function of CXCR4 in cardiac remodeling after MI in genetically modified mice ($CXCR4^{+/-}$) in order to identify possible adverse effects that pharmacological compounds may have (Chapter II.6.). The results showed that early EPCs secrete increased amounts of SDF-1 and are able to bind SDF-1 to their surface, thus creating a local gradient that may attract from circulation stem/progenitor cells $CXCR4^{+}$ with angio-/vasculogen potential. On the other hand, *in vivo* studies on transgenic mice demonstrate that CXCR4 has a double-edged effect on cardiac remodeling after MI. Thus, on one hand, mice $CXCR4^{+/-}$ presented smaller and more stable scars after IM, this associated with rather regenerator monocytic response and better adaptation of cardiac myocytes to hypoxic stress. On the other hand, early EPC function is impaired in transgenic mice, reduced myocardium neovascularization and the reduction of coronary flow recovery, being responsible for the lack of improvement of ventricular function. Thus, extrapolating these results to inhibit CXCR4 in human system should be done with caution.

In conclusion, the data contained in this thesis demonstrates that:

- Early and late EPC can be obtained from peripheral blood and cord blood

- Endothelial potential of stem/progenitor mesenchymal cells is limited, but this cell type can be exploited to stimulate neovascularization based on their paracrine properties

- Factors released *in vitro* by early EPC are able to significantly stimulate the formation of new vessels by late EPC, early EPC transplantation is not mandatory to achieve this effect.

- Early EPC maintains their paracrine properties under hypoxia and VEGF is not essential for their angiogenic effect.

- Paracrine factors secreted by MSC supports some of the processes involved in angiogenesis (support chemotaxis and adherence of EC, but not proliferation) and have beneficial effects on ischemic cardiomyocytes, and these properties are maintained when MSC are subject to hypoxia.

- *In vitro* effects of early EPC are complementary to those induced by MSC, stimulating proliferation, but not EC adhesion. Both of these effects can be induced simultaneously by combining the conditioned medium (obtained from the EPC and MSC). Thus, the combination of these two cell populations or factors secreted by them could be a successful strategy for inducing therapeutic angiogenesis.

- Early EPC secrete increased amounts of SDF-1 and are able to bind SDF-1 to their surface, thus creating a local gradient that can attract circulating stem/progenitor cells $CXCR4^{+}$ with angio-/vasculogenic potential to an ischemic site.

- CXCR4 presents a double-edged effect on myocardial remodeling after MI, its low expression causing the formation of smaller and more stable scars, but affecting cardiac neovascularization and recovery of ventricular function.

Based on the original results obtained during the doctoral internship new strategies can be envisaged that might optimize the therapy for revascularization of an ischemic territory. These preclinical data are likely to be strengthened in future clinical trials.

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