New Therapies for Myocardial Infarction

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Myocardial infarction

Cardiovascular disease is a leading cause of morbidity and mortality in the western world, with 17.5 million deaths worldwide in 2005, representing 30 % of all global deaths that year. Acute myocardial infarction (AMI), commonly known as a heart attack, plays a major role in this setting. AMI caused 7.6 million deaths worldwide in 2005, therewith representing 13% of all male deaths and 12% of all female deaths that year.[1,2] AMI usually results from plaque rupture with thrombus formation in a coronary vessel, depriving the myocardium from blood, mostly resulting in myocardial necrosis. Classical symptoms of AMI include sudden chest pain (typically radiating to the left arm), shortness of breath, nausea, vomiting, palpitations, sweating and anxiety.[3] Important risk factors for developing AMI are previous cardiovascular disease, increased age, smoking, hypercholesterolaemia, hypertension, obesity, chronic kidney disease and excessive alcohol consumption.[4-6]

Prognosis for AMI varies greatly, depending on a person's health, on the extent of heart damage and on the efficacy of the treatment given. AMI can result in heart failure, independent of treatment, and sometimes also in death. For the period 2005 - 2008 in the United States the median mortality at 30 days post-AMI for patients from 65 years and older was 16.6%.[7,8] Heart failure is mainly caused by the loss of cardiomyocytes, as spontaneous regeneration of the cardiomyocytes in the heart is limited. As a results of this, injured myocardium is replaced by scar tissue, which adversely affects cardiac function.[1,9] Therapy for AMI mainly focuses on restoration of vascular perfusion (called reperfusion), for example through thrombolysis, percutaneous coronary intervention, or coronary artery bypass surgery, and on prevention of secondary cardiovascular events via specific drug therapy, like for instance ACE-inhibitors and beta blockers.[10,11] Although these strategies significantly reduce mortality,[12,13] they do not replace lost cardiomyocytes. The ideal AMI therapy would therefore be to stimulate revascularization of the ischemic region, to minimize the loss of cardiomyocytes resulting in reduction of scar tissue formation, and finally the regeneration of cardiomyocytes.[11]

Inflammation and inflammation inhibitors

As stated above, a major focus of current AMI treatment is reperfusion of the ischemic myocardium. Reperfusion, however, also results in the induction of inflammation in the heart. Through the binding of the inflammatory mediators C-reactive protein (CRP) and complement on the cell membrane of injured and dead cardiomyocytes, these cells are then cleared from the heart. At the same time, this inflammation has been shown to induce additional damage to the myocardium.[14-16] Ischemia namely not only results in areas of irreversibly damaged (necrotic) cardiomyocytes. In the border zones of these necrotic areas also morphologically normal cardiomyocytes can be found that are reversibly damaged. Inflammation then also targets these reversibly damaged cardiomyocytes in the border zones resulting in an increase of the initial infarction area.[17] Therefore inhibition of this inflammation might form a potent new target of therapy. However, it has to be emphasized that although inflammatory reactions can cause additional damage to the myocardium, the inflammation response itself is also essential for the healing process of the heart after myocardial infarction.[18] As such it was shown that administration of corticosteroids to patients with AMI, delayed scar tissue formation in the heart due to

inhibition of the general inflammatory response, resulting in cardiac rupture.[19,20] Therefore, it is of utmost importance that anti-inflammatory therapy after AMI only rescues reversibly damaged cells and does not affect normal wound healing. PX-18, an inhibitor of type IIA secretory-type phospholipase A_2 ($sPLA_2$-IIA) and clusterin theoretically would have such an effect.[15,21]

$sPLA_2$-IIA is an acute phase enzyme that belongs to the family of phospholipases A_2 (PLA_2).[22] Previous work in our group has shown that during reperfusion $sPLA_2$-IIA binds to and induces cell death of both necrotic, but also reversibly damaged cardiomyocytes.[17,23] $sPLA_2$-IIA namely facilitates binding of CRP to cardiomyocytes. This binding then induces binding and activation of complement, resulting in further tissue damage.[24] In addition, $sPLA_2$-IIA not only induces cell death through activation of complement, but also via a direct cytotoxic effect itself.[23] Inhibition of $sPLA_2$-IIA would thus form an ideal therapeutic target. More important, as it was found that CRP can bind to necrotic cells, but not reversibly changed cells in the absence of $sPLA_2$-IIA,[24] inhibition of $sPLA_2$-IIA theoretically thus would only reduce death of reversibly damaged cardiomyocytes, without impairing normal wound healing. In this thesis we investigated this hypothesis using PX-18, a specific $sPLA_2$-IIA inhibitor, *in vitro* and *in vivo* using a rat model of AMI. (Chapter 2)

Clusterin, or apolipoprotein J, a 75-80 kDa secreted protein, is widely expressed in mammalian tissues by different cell types and in most body fluids, including blood. It was shown that clusterin inhibits several factors of the complement system.[25-27] Depositions of clusterin have also been described in the heart after AMI, where it was shown to bind to cardiomyocytes.[28,29] Clusterin then was not only found on irreversibly damaged cardiomyocytes in necrotic areas of the human heart after AMI, but interestingly also on morphologically viable, complement-negative cardiomyocytes in the border zones of the human infarcted heart. *In vitro* it was found that addition of purified human clusterin protected ischemic challenged rat cardiomyocytes against cell death, interestingly also in the absence of complement.[21] As proof of principle we studied whether application of purified human clusterin would also reduce myocardial damage in an *in vivo* rat model of AMI. (Chapter 3)

Stem cells

Although current therapies in AMI patients significantly reduce mortality,[30,31] they do not replace lost cardiomyocytes as stated above. As such, stem cell therapy might replace lost cardiomyocytes after AMI, since stem cells can differentiate into cardiomyocytes.[1,32,33] Stem cells are primal cells, common to all multi-cellular organisms, that retain the ability to renew themselves through cell division and to differentiate into a wide range of specialized cell types. Two broad categories of human stem cells exist, namely human embryonic stem cells and adult stem cells. In a developing embryo, stem cells are able to differentiate into all of the specialized embryonic tissues. Therefore, in theory these embryonal stem cells are very appealing for therapy because of their pluripotentiality. However, the use of embryonic stem cells is limited due to putative teratoma formation, ethical problems, and the lack of possible autological transplantation.[9] When however adult stem cells are used, theoretically a patient could be transplanted with his or her own stem cells. Adult stem cells are undifferentiated cells that can be found in a number of organs in the body.[11,34] Recent studies indicate that within adult stem cells, subpopulations of mesenchymal stem cells have the ability to transdifferentiate *in vitro* to cells of

mesenchymal as well as non-mesodermal lineages, with the appropriate stimuli and environmental conditions.[34] As such, adult stem cells form a promising therapy, for example in AMI.

However, to effectively restore contractile function of the heart after AMI, sufficient stem cells need to attach to the infarcted area, and subsequently proliferate and differentiate into cardiomyocytes. Unfortunately, recent studies suggest that only a small proportion of mesenchymal stem cells applied to the heart invade the infarcted area and finally differentiate into cardiomyocytes. Furthermore, most of the applied stem cells die within the first week after transplantation.[1,35]. Thus although stem cell therapy is very promising, before further translation to the clinical setting, stem cell therapy needs to be further optimized.

Adipose tissue derived stem cells

Stem cells of different sources have been used experimentally for transplantation after AMI, although the optimal choice of stem cell source remains controversial. Many studies have been conducted using bone marrow mesenchymal stem cells. Although these cells are promising candidates for myocardial regeneration after AMI, the yield of a cell isolation is low, and isolation can be painful.[36] Another potential source of stem cells is adipose tissue. The stromal vascular fraction of adipose tissue (SVF) contains adipose derived stem cells (ASCs). ASCs are of mesenchymal origin, and have properties similar to bone marrow mesenchymal stem cells as has been shown *in vitro* and *in vivo*[34,36]. These ASCs can be easily harvested, show high proliferation rates in culture and have the capacity to differentiate towards several cell types, amongst which cardiomyocytes.[37-39] Even more, when compared to bone marrow, adipose tissue provides up to 100 times more mesenchymal stem cells per gram tissue.[36,37] Regarding this high yield of stem cells, theoretically also uncultured SVF cells can be used for transplantation, since this population contains high numbers of stem cells making culturing unnecessary. Culturing namely is time consuming and expensive, and as such is disadvantageous for clinical practice.[37] Even more, culturing may also affect the functional characteristics of stem cells.[36,40] Taken together, ASCs are a promising source of stem cells, however studies on human ASCs are limited. In this thesis we therefore have studied characteristics of human ASCs, investigated factors that might influence the fate of these cells when used for therapy after AMI, and finally analyzed the therapeutic potency of ASCs.

Expression of Multi Drug Resistance proteins on stem cells

A relative unknown intrinsic mediator of ASCs that might influence the fate of stem cells after AMI is the expression of multidrug resistance (MDR) proteins. MDR proteins are broad specificity ATP-binding cassette transporters, which mediate multidrug resistance in mammalian cells by excretion of cell toxic substances. Expression of MDR proteins is a widely accepted characteristic of stem cells, also in ASCs.[41,42] In general, MDR proteins were suggested to have an anti-apoptotic function in stem cells, but this has not been investigated in ASCs.[43,44] P-glycoprotein-1 (PGP-1) and breast cancer resistance protein-1 (BCRP) have been widely investigated in stem cells, and have been shown to be associated with the efflux of Hoechst dye, which is a known characteristic of haematopoietic stem cells and stem cells in general.[45,46] Both these proteins have been shown to play a role in removing a broad range of

damaging substances and metabolites from cells in which they are expressed.[41,43] Interestingly, BCRP was also found to improve survival of hematopoietic stem cells during hypoxia. However, studies describing a protective effect of MDR proteins in stem cells during hypoxia or ischemia are scarce, and this protective effect has not been investigated for ASCs.[44] Until now, most research on MDR proteins in stem cells has been performed on haematopoietic stem cells. For ASCs, only BCRP but not PGP expression was shown, without further studies on the functional role of this protein.[47,48] We hypothesized that ASCs with high expression levels of MDR proteins might have higher survival chances when transplanted in the ischemic and inflammatory episode after AMI. We therefore investigated the expression and activity of BCRP and PGP in ASCs, and studied whether these proteins protected ASCs during ischemia. (Chapter 4)

Extracellular matrix

Homing, growth and differentiation of stem cells after AMI is influenced by the heart itself, related to adhesion factors and the extracellular matrix (ECM) at the site of injury, that changes dramatically after AMI.[1,49,50] Two important extracellular matrix (ECM) molecules, that have been associated with human mesenchymal stem cell survival and differentiation, are fibronectin and laminin.[51-53] However, these proteins have not been studied in relation with cardiomyocyte differentiation of stem cells.

Fibronectin is a large molecular weight glycoprotein, present at low levels in the ECM of the healthy heart.[54,55] It was demonstrated that fibronectin enhances the attachment of bone marrow derived stem cells *in vitro*, compared to several other ECM molecules.[56,57] Furthermore, it was shown that fibronectin can influence stem cell behaviour such as migration and proliferation, *in vitro* as well as *in vivo.*[53,58-60] Laminins are heterotrimers of large polypeptides, which are mainly localized in the basement membrane of the cardiomyocytes in the heart.[61,62] It is known that laminin plays a role as principle adhesive molecule for maintenance of architecture and differentiation in the embryo.[61] In addition, several studies have described a positive effect of laminin on myogenesis.[62-64] Both laminin and fibronectin are expressed in the normal heart, and were found to be increased after AMI, however the exact localization of these proteins during different phases of AMI was not studied before.[54,65,66] We hypothesized that these ECM molecules would have an effect on ASC survival and differentiation. We therefore analyzed their expression pattern in time and their functional effect on ASCs, to construct the ideal time window for ASC transplantation after AMI. (Chapters 5 and 6)

Timing of stem cell transplantation

Next to the ECM, also induction of acute inflammation post AMI might influence the ideal time window to apply stem cell therapy. It is namely known that up to 5 days post AMI, extensive depositions of complement and CRP are found in the heart, enhancing local inflammatory reactions.[67] This theoretically not only could be harmful for cardiomyocytes, but also for implanted stem cells.[50]

It is thus important to apply stem cell therapy at the moment after infarction when the environment is most favourable for stem cell adhesion and cardiomyocyte formation. However, to the best of our knowledge, this ideal time frame of stem cell injection has yet not been determined. In most animal studies, investigators apply stem cells during the same operational procedure as the infarct

induction, i.e. directly after infarct induction. Albeit, recent studies suggest that later time points might be more favourable for the survival of the stem cells.[1,50,68] In this thesis we therefore studied the therapeutical effect and the optimal transplantation time point of ASCs after infarction in a rat model of AMI, and compared freshly isolated SVF cells with cultured ASCs herein. (chapter 7)

Aim of this study

The aim of this thesis was to develop and improve new putative therapies for myocardial infarction. We first studied the effect of two unique inflammatory inhibitors (PX-18 and clusterin), which might specifically save reversibly damaged cells, without affecting normal wound healing, on cardiac outcome after infarction. Next we tried to optimize stem cell therapy, since stem cell therapy forms a promising regenerative therapy in AMI. We focused on ASCs, as these cells were found to be promising candidates for stem cell therapy, due to their relatively high numbers and easy harvesting procedure in the human body, combined with their pluripotency. We studied the therapeutic potency of these ASCs at three different levels, namely: 1) intrinsic factors of the stem cells that affect their functionality, 2) the role of the extracellular matrix on stem cell binding/differentiation properties, and 3) comparison of freshly isolated SVF cells with cultured ASCs, including an analysis of the optimal timepoint of stem cell transplantation after AMI.

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