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Hypoxia-Inducible Factor 1-Alpha Release After Intracoronary Versus Intramyocardial Stem Cell Therapy in Myocardial Infarction

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Abstract We have investigated the effect of stem cell delivery on the release of hypoxia-inducible factor 1 alpha (HIF-1α) in peripheral circulation and myocardium in experimental myocardial ischemia. Closed-chest, reperfused myocardial infarction (MI) was created in domestic pigs. Porcine mesenchymal stem cells (MSCs) were cultured and delivered ($9.8\pm1.2\times10^6$) either percutaneously NOGA-guided transendocardially (Group IM) or intracoronary (Group IC) 22±4 days post-MI. Pigs without MSC delivery served as sham control (Group S). Plasma HIF-1α was measured at baseline, immediately post- and at follow-up (FUP; 2 h or 24 h) post-MSC delivery by ELISA kit. Myocardial HIF-1α expression of infarcted, normal myocardium, or border zone was determined by Western blot. Plasma level of HIF-1α increased immediately post-MI (from 278±127 to 631±375 pg/ml, $p<0.05$). Cardiac delivery of MSCs elevated the plasma levels of HIF-1α significantly ($p<0.05$) in groups IC and IM immediately post-MSC delivery, and returned to baseline level at FUP, without difference between the groups IC and IM. The myocardial tissue HIF-1α expression in the infarcted area was higher in Group IM than in Group IC or S (1,963±586 vs. 1,307±392 vs. 271±110 activity per square millimeter, respectively, $p<0.05$), while the border zone contained similarly lower level of HIF-1α, but still significantly higher as compared with Group S. Trend towards increase in myocardial expression of HIF-1α was measured in Group IM at 24 h, in contrast to Group IC. In conclusion, both stem cell delivery modes increase the systemic and myocardial level of HIF-1α. Intramyocardial delivery of MSC seems to trigger the release of angiogenic HIF-1α more effectively than does intracoronary delivery.

Keywords Hypoxia-Inducible Factor 1 Alpha · Myocardial Infarction · Stem Cell · Cardiac Delivery

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Introduction

The stem cell (SC)-based cardiac regeneration offers a new mode of cardiovascular therapy for patients with ischemic heart disease [1–6]. Preclinical [7–9, 12] and clinical trials [10, 11] have proven the safety, feasibility, and modest efficacy of the cardiac SC delivery. Preclinical studies have revealed SC infiltration in the damaged myocardium with increased microvascularity [7, 12] and also newly formed myocardium in the infarcted part of the left ventricle after transplantation of bone marrow (BM) origin SCs. Beside various potential mechanisms, such as plasticity or cell fusion [13], the paracrine effect of delivered SC has been suggested, involving release or production of many angiogenic proteins, such as vascular endothelial growth factor (VEGF), angio-

genic cytokines or other growth factor, or nitric oxide (NO). However, in spite of extensive research, the main mechanism of cardiac repair by SC is still unknown.

Hypoxia-inducible factor-1 (HIF-1) is a heterodimeric α/β transcription factor that mediates tissue responses to hypoxia [14]. HIF-1 promotes transcription of more than 60 genes involved in oxygen homeostasis in response to reduced oxygen tension, including inducible NO synthase (iNOS), VEGF, and heme oxygenase-1 [15]. HIF-1α plays a critical role in a variety of physiological processes, ranging from tissue remodeling, metabolism, glycolysis, erythropoiesis, cell proliferation, and cell survival to myocardial angiogenesis [16]. Naked plasmid DNA encoding modified HIF-1α/VP16 has been shown to improve perfusion in a rabbit model of hindlimb ischemia [17]. HIF-1α and HIF-1-beta mRNAs are expressed in most human tissues [18], but HIF-1 activity is determined by expression and activity of the α subunit [19]. Under normoxic conditions, HIF-1α subunits have an exceptionally short half-life (~3–5 min) and low steady-state levels [20], and are degraded by the 26S proteasome (Prolyl 4-Hydroxylase-2, PHD2) [21–23]. It has recently been demonstrated that the inhibition of HIF-1α degradation through shRNA knockdown of PHD2 in the ischemic mouse heart offers a novel angiogenic therapy approach [24].

Dekel et al. reported vasculogenesis after transplantation of human hematopoietic SCs into ischemic and growing kidneys, after rapid and prolonged induction of stromal-derived factor (SDF)-1 and HIF1 mRNA, which enhanced engraftment of human CD34+ cells [25]. As compared with skeletal myoblasts alone, the coadministration of HIF 1α resulted in a significantly greater degree of angiogenesis, cell engraftment, and cell survival [26]. Moreover, HIF-1α, a transcriptional regulator of VEGF gene expression, was found to be expressed in cultured SCs and in putative SCs in sections of in vivo stretch-injured rat muscle. Hypoxic culture conditions increased SC HIF-1α activity, which was positively associated with SC VEGF gene expression and protein levels [27]. Interestingly, no data exist on local myocardial expression and systemic release of HIF-1α after cardiac SC therapy.

Accordingly, the aim of our study was to investigate the local myocardial expression of HIF-1α 2 h and 24 h after cardiac stem cell delivery and the release of HIF-1α into systemic circulation in response to intracoronary or intramyocardial SC therapy in the porcine model of chronic myocardial ischemia.

Methods

BM Harvesting and MSC Selection

BM was harvested by aspiration of 100 mL BM from the iliac crest of five pigs and stored at 4°C in a Baxter bag (Baxter Healthcare, Ltd, Thetford, Norfolk, UK). The MSCs were selected by Ficoll-Paque gradient (Ficoll-Paque, Amersham Biosciences). Buffy coats were plated at 50,000 cells per square centimeter in α MEM medium without nucleotides, and containing 10% fetal calf serum and 2 mM L-glutamine, penicillin/streptomycin supplemented with 1 ng/ml fibroblast growth factor 2. Cells were harvested by trypsinization when 75% confluent and replated at a cell density of 1,000 cells per square centimeter. The prepared cells were negative for CD45 expression.

Differentiation and Characterization of the MSCs Stemness

The differentiation media for adipogenesis consisted of DMEM (low glucose), 20% FCS, 0.5 mM isobutylmethylxantine, 60 μM indomethacin, and 10^6 M dexamethasone. For osteoblastic differentiation, the medium consisted of DMEM (high glucose), 10% FCS, 10 mM bet-glycerophosphate, 50 μg/ml L-ascorbic acid, and 10-7 M dexamethasone. For chondrogenic differentiation, a micropellet system was used. Cells were suspended in DMEM, containing 0.1 μM dexamethasone, 1 mM sodium pyruvate, 0.17 mM ascorbic acid, 0.35 mM proline, and 1:500 stock from Cambrex of insulin-transferrin-selenium. The medium was supplemented with bone morphogenic protein-2 at 100 ng/ml or TGF-β at 10 ng/ml. The differentiation of the MSC to the chondrogenic, adipogenic, and osteogenic cell lineage were tested by staining of the cells with alcian blue (staining of proteoglycans), oil red O (staining of intracellular lipid) and von Kossa (staining of mineralized calcium), respectively, after incubation in the corresponding induction medium.

Induction of AMI in Pigs

All animal studies were approved by the local Experimental Animal Care Committee of the University of Kaposvar, Hungary, where the studies were performed. Closed-chest, reperfused acute myocardial infarction (AMI) was induced in 22 domestic pigs (27±3 kg) by percutaneous occlusion of the left anterior descending coronary artery (LAD). After overnight fasting, the pigs (weight 18–30 kg) were sedated with 12 mg/kg ketamine hydrochloride, 1.0 mg/kg xylazine, and 0.04 mg/kg atropine. Following intratracheal intubation, the anesthesia was deepened with isofluran and O_2 via a mask. Intratracheal intubation was then performed to maintain the anesthesia with 1.5–2.5 vol% isofluran, 1.6–1.8 vol% O_2, and 0.5 vol% N_2O. During anesthesia, the O_2 saturation and ECG were monitored continuously. After the administration of 200 IU/kg of heparin, selective angiography of the left coronary tree was performed, and a balloon catheter (3.0 mm in diameter ×15 mm long, Maverick, Boston Scientific Corp, Natick, MA, USA) was advanced into the LAD after the origin of the first major

diagonal branch. The LAD was then occluded by inflation of the balloon at 5 atm for 90 min, followed by balloon deflation to allow reperfusion. The pigs were subsequently allowed to recover.

Three weeks after AMI creation, the pigs were randomized and received either an intracoronary infusion (Group IC, $n=11$) or intramyocardial injections (Group IM, $n=8$) of the MSC, while coronary angiography without MSC administration was performed in sham-control animals (Group S, $n=3$).

Assessment of Left Ventricular Function

The global left ventricular (LV) function was controlled by means of transthoracic echocardiography at baseline and post-reperfusion. The LV end-diastolic (EDV), end-systolic (ESV), and stroke volumes (SV) and the global ejection fraction (EF) were measured by using the area-length method.

Intracoronary and Intramyocardial Delivery of MSCs

Following placement of a 6F sheath (Terumo Medical Corporation) and angiography of the LAD, a guiding catheter was introduced into the ostium of the infarct-related artery. A Concerto semi compliant over the wire balloon and infusion catheter (OCCAM International BV, The Netherlands) was introduced into the LAD over an appropriate guidewire. The guidewire was then withdrawn, and the heparin-diluted cell suspension of MSCs was slowly injected over about 15 min using the stop-flow technique (Fig. 1). The patency of the target vessel after the injection was confirmed angiographically.

For intramyocardial delivery, an 8F sheath (Terumo Medical Corporation) was placed in the femoral artery, and a diagnostic NOGA catheter (Cordis, Johnson & Johnson, Miami Lakes, FL, USA) was advanced into the LV cavity. Detailed descriptions of the endocardial mapping system components were published previously [28–30]. After the diagnostic NOGA endocardial mapping, the MSCs were injected into the peri-infarct myocardium at five to six sites using a Myostar injection catheter (Cordis, Johnson & Johnson; Fig. 1). The injections (0.3 ml cell suspension each) were given slowly (30 to 40 s) and only into areas with a unipolar voltage above 5 mV, using the quality control criteria [31].

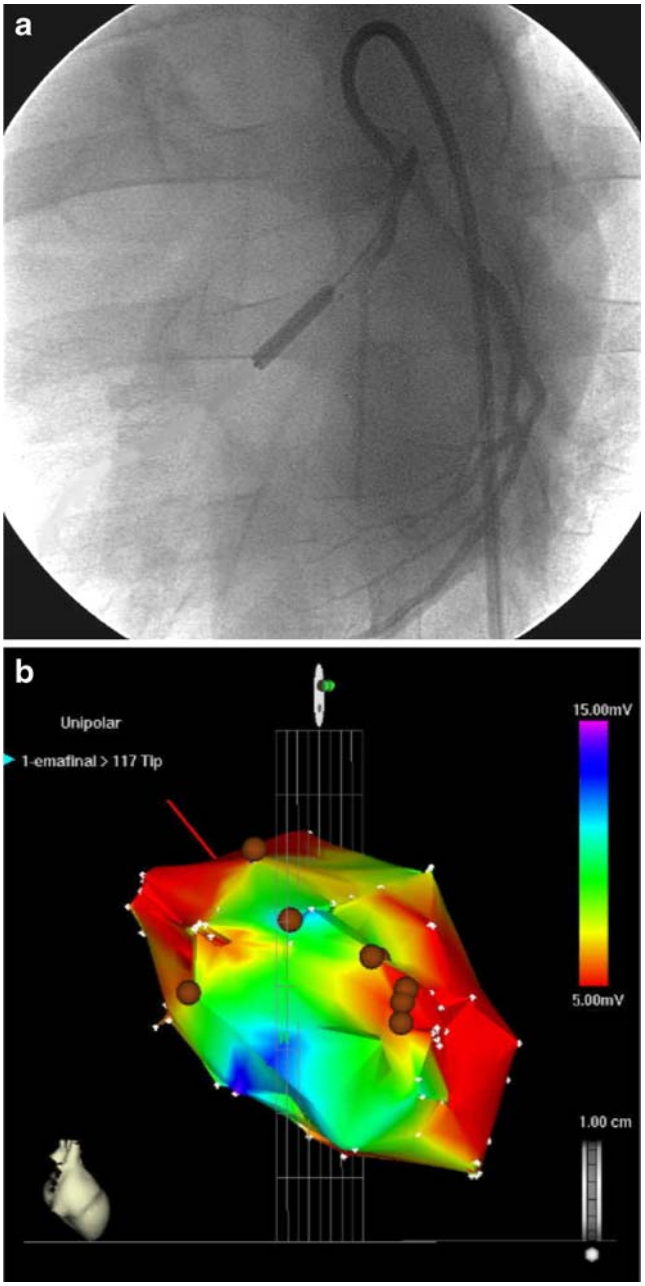

Fig. 1 Intracoronary (**a**) and intramyocardial (**b**) delivery of mesenchymal stem cells. Representative NOGA endocardial mapping with intramyocardial injections (*brown points*) administered in the border zone of infarction (*yellow*). No injections were made in the infarcted area (*red*) or in the normal myocardium (*blue and pink*)

Plasma and Myocardial Tissue Concentration of HIF-1α

Blood samples were taken before induction of AMI, immediately post-AMI before reperfusion of the infarct-related artery and 2 h post-AMI.

Additionally, blood samples were collected before MSC delivery, 1 h post-SC delivery and at FUP (either at 2 h or 24 h post-MSC administration) or sham delivery in all groups. The plasma HIF-1α concentration was measured with a commercially available porcine HIF-1α ELISA kit (Cusabio Biotech Co Ltd, Newark, DE, USA).

Myocardial tissue samples were taken from the infarcted area, the border zone of the infarction, or the normal myocardium. The heart tissue was homogenized (Ultra Turrax T8; 20,000/min, 2×30 s) on ice in TRIS–mannitol

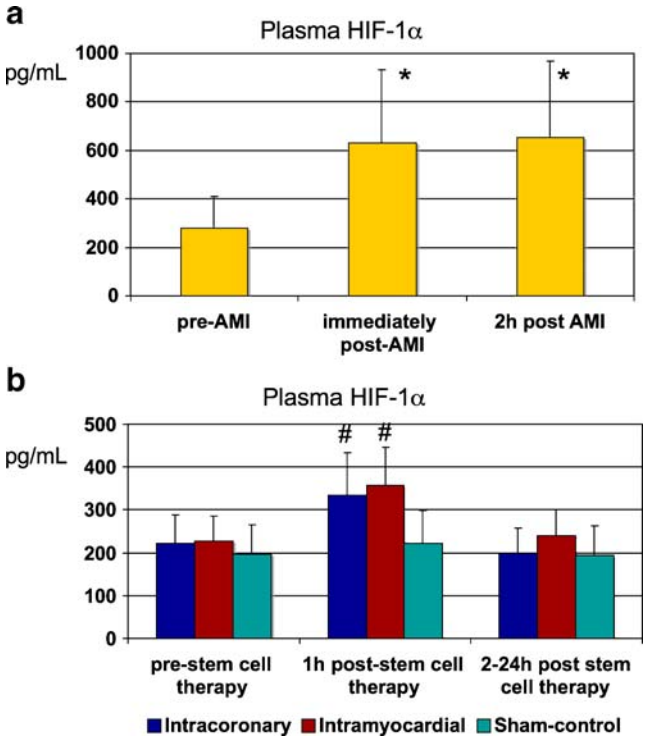

Fig. 2 Plasma HIF-1α release pre-, immediately after, and 2 h post-infarction (**a**). *$p<0.05$ between baseline level and immediately post-AMI or 2 h post-AMI. Plasma HIF-1α release before, 1 h post-, and 2–24 h after intracoronary or intramyocardial stem cell delivery or in sham control (**b**). #$p<0.05$ between baseline level and 1 h post-stem cell delivery

buffer (pH 7.4) containing 2.0 mM TRIS, 50.0 mM mannitol, 100.0 μM phenylmethylsulfonyl fluoride, 12.0 μM leupeptin, 0.5 mU/ml aprotinin, and 0.5% Triton X-100. Cellular debris was pelleted by centrifugation at 12,000 rpm for 20 min at 4°C. Aliquots of 25 μg of total cellular protein were denatured by mixing and boiling with 20.0 mM TRIS, 3.0 mM EDTA, 2% SDS, 10% mercaptoethanol, 20% glycerol, and a trace amount of bromophenol blue. Equal amounts of protein samples were electrophoresed (100 V) in 10% SDS-PAGE gel. After electrophoresis, the protein was electrophoretically transferred from the unstained gel to a nitrocellulose membrane (Amersham, Pharmacia Biotech., Buckinghamshire, UK). The blots were probed with the primary HIF-1α monoclonal antibody at 1:5,000 dilution (Abcam, Cambridge, UK). The HRP-conjugated secondary antibody was used at 1:2,000 dilution (Biotechnology Inc., Santa Cruz, USA), and the immunoreactive bands were visualized by using the ECL Advance Western Blotting Detection Kit (Amersham Biosciences).

Statistics

Continuous parameters were expressed as means±standard deviation. Differences between the plasma HIF-1α concentrations of the groups were analyzed by using analysis of variance supplemented with the unpaired *t* test. The correlation between the injected MSC number and the plasma or myocardial level of HIF-1α was calculated by linear regression analysis. A difference with $p<0.05$ was considered statistically significant.

Results

The global LV EDV, ESV, SV, and EF were 80.3±10.4 ml, 55.1±7.5 ml, 25.2±6.0 ml, and 31.2±5.4% 3 weeks post-AMI, respectively, with no difference between the groups or within the groups pre-SC or 2–24 h post-SC delivery.

Plasma level of HIF-1α increased immediately post-MI (from 278±127 to 631±375 pg/ml, $p<0.05$) and remained high during the 2 h reperfusion (653±361 pg/ml) in all animals (Fig. 2).

Cardiac delivery of MSCs increased the plasma levels of HIF-1α significantly ($p<0.05$) in groups IC (from 222±80 to 334±62 pg/ml) and IM (from 228±88 to 358±134 pg/ml) 1 h post-MSC delivery and returned to baseline level at 2–24 h (199±68 vs 240±114 pg/ml in groups IC vs IM) without difference between the groups. The plasma level of HIF-1α increased slightly during angiography in Group S (from 197±48 to 222±68 at 1 h and 195±55 at 2 h; Fig. 2).

The myocardial tissue HIF-1α expression of the infarcted area was higher in Group IM than in Group IC or Group S (1,963±586 vs. 1,307±392 vs. 271±110 activity per square millimeter, respectively, $p<0.05$), while the border zone contained a similarly smaller level of HIF-1α in

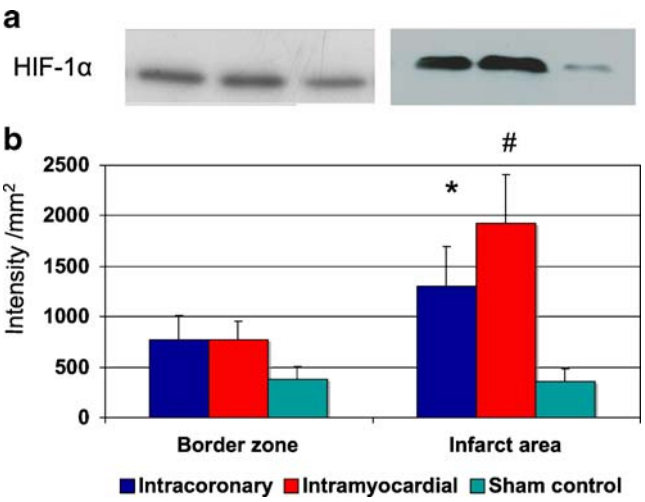

Fig. 3 Western blot analysis of myocardial HIF-1α expression (**a**). Myocardial expression of HIF-1α in the border zone and the infarcted area after intracoronary or intramyocardial delivery of MSC and in sham control (**b**). *$p<0.05$ between intracoronary delivery and sham control. #$p<0.05$ between intramyocardial delivery and intracoronary delivery or sham control

Table 1 Time-dependent myocardial expression of HIF-1α after intracoronary (Group IC) or intramyocardial (Group IM) administration of MSC or sham control (Group S) in porcine chronic ischemic heart

Group	FUP time	Number of injected MSC	HIF-1α expression border zone (activity/mm^2)	HIF-1α expression infarct area (activity/mm^2)
Group IC	2 h (n=7)	10.9±1.2×10^6	1,046±91	1,428±365
	24 h (n=4)	10.9±1.1×10^6	736±336	1,290±507*
Group IM	2 h (n=4)	9.4±1.0×10^6	834±97	1,674±387
	24 h (n=4)	10.7±1.6×10^6	700±319	2,172±698*
Group S	2 h (n=3)	0	355±92**	271±110**

*p<0.05; **p<0.05 between Group S and Group IC or IM

groups IC and IM (776±335 and 767±230 activity per square millimeter), but still significantly higher than in Group S (355±92 activity per square millimeter; Fig. 3).

No significant correlation was found between the number of injected cells and myocardial or plasma HIF-1α level.

Subanalysis revealed an obvious trend towards further increase in myocardial expression of HIF-1α in Group IM at 24 h, which was not observed in Group IC (Tables 1 and 2).

Discussion

Our study demonstrates the significantly higher level of local expression of angiogenic HIF-1α in the infarcted myocardium after the cardiac delivery of MSCs. Intramyocardial delivery seems to be more effective than intracoronary administration, probably because of the higher cell retention. The level of HIF-1α in the systemic circulation was also elevated post-MSC delivery, independently of the delivery mode.

HIF-1α in Ischemia–Reperfusion

Similarly to others [32, 33], we found that ischemia/reperfusion increased both the plasma and myocardial tissue levels of HIF-1α. Myocardial hypoxia is a well-known potent inducer of HIF-1α protein expression [34], and the combination of a reduced oxygen tension and the activation of Erk1/2 signaling enhances HIF-1α expression and activity [35]. Furthermore, HIF-1α upregulates transcription of VEGF [36]. Both of these important angiogenic proteins play a central role in coronary collateral development in response to chronic myocardial ischemia [37].

Interestingly, the plasma level of HIF-1α was somewhat higher after prolonged anesthesia during induction of infarction or MSC delivery, as compared with the single blood sampling, when only short anesthesia was introduced. These findings are in accordance with the fact, that volatile anesthesia with isoflurane enhances the release of HIF-1α [38], and contributes to pharmacologic preconditioning during anesthesia. However, the obvious changes between the pre- and post-MI and pre- and post-MSC administration as regards the plasma HIF-1α concentrations, and the significant increase in HIF-1α expression in Group IM as compared with group IC is convincing, that these changes are not only the consequences of the isoflurane-induced HIF-1α release.

Effect of MSC Delivery on Myocardial Expression of HIF-1α

HIF factors regulate a variety of genes that affect a myriad of cellular processes including metabolism, angiogenesis, cell survival, and oxygen delivery, all of which are important in the heart. In humans, Lee et al. [39] have found

Table 2 Time-dependent release of HIF-1α in systemic circulation after intracoronary (Group IC) or intramyocardial (Group IM) administration of MSC or in sham control (Group S) in porcine chronic ischemic heart

Group	FUP time	Plasma HIF-1α pre-delivery (pg/ml)	Plasma HIF-1α 1h post-delivery (pg/ml)	Plasma HIF-1α at FUP (pg/ml)
Group IC	2 h (n=7)	186±58	323±71	210±51**
	24 h (n=4)	251±80	369±28	197±40**
Group IM	2 h (n=4)	218±102	443±102	355±78*
	24 h (n=4)	246±97	310±132	163±31***
Group S	2 h (n=3)	197±48	222±101	195±51**

*p<0.05 Group IM 2 h; **p<0.05 Group IC 2 h or S 2 h; ***p<0.05 Group IM 24 h

increased HIF-1α in myocardial peri-ischemic zone of patients undergoing CABG. Similarly, we observed an increased myocardial expression of HIF-1α in the infarcted myocardium and even though to a lesser extent, in the border zone of infarction, which might have an important role as concerns MSC engraftment and angiogenic potential. Dekel et al. reported the improved homing of human SCs in injured kidneys, a process likely to be dependent on local signals induced by ischemia, including SDF1 and HIF-1α, which in turn affect CD34+ cell migration and function [25]. In the experiment of Rhoads et al. the increased SC HIF-1α activity was positively associated with SC VEGF gene expression and protein levels [27]. HIF-1α upregulates VEGF transcription by binding to specific promoter sequences and preserves VEGF translation during hypoxic conditions by enhancing mRNA stability [40]. Thus, the HIF-dependent gene expression could potentially modify the cell survival in myocardial ischemia and reperfusion [42].

In our experiment we have used the chronic infarction model; therefore, enhanced endogenous recruitment of autologous hematopoietic stem cells in myocardial infarcted area was not expected. However, the implanted allogeneic MSC-induced systemic release of HIF-1α and the enhanced myocardial expression of HIF-1α might positively act on systemic or local VEGF release and further autologous stem cell recruitment, which might multiply the regenerative process. This concept is confirmed by Chang et al. pointing out that increased degradation of HIF-1α in aged mice resulted in reduced activation of the hypoxia response genes VEGF and SDF-1, and diminished the chemotactic signal to endothelial progenitor cell recruitment with impaired neovascularization and increased tissue necrosis [41].

MSCs are known to be able to produce HIF-1α themselves. In the absence of labeling of the MSCs, it is questionable whether the increased HIF-1α expression in the myocardium is the product of MSCs or the cardiomyocytes. The myocardial expression of HIF-1α was further increased at 24 h post-MSC intramyocardial delivery, which was not observed after intracoronary delivery of MSC. The higher level of HIF-1α in the infarcted area might be explained by the HIF-1α production by survival myocytes, or by the MSCs migrating into the infarcted area. Intramyocardial delivery of the MSC led to a higher level of HIF-1α expression, probably due to a higher level of cell retention [5].

Immunogeneic Effect of Transplantation of Allogeneic MSCs on Myocardium

Graft-versus-host disease is the most frequent (up to 50%) complication after allogeneic bone marrow or hematopoietic SC transplantation. It is associated with considerable morbidity and mortality, in spite of many approaches to therapy with increasing dose of steroids, addition of polyclonal or monoclonal antibodies, use of immunotoxins, or additional immunosuppressive/chemotherapeutic interventions. MSCs have recently been shown to mediate immunomodulatory properties in vitro and in vivo, interacting with cellular components of the immune system and inducing a shift from pro- to anti-inflammatory cytokines by inhibiting the proliferation of all kinds of T-, B-, and NK-cells [43]. Accordingly, co-transplanting of ex vivo expanded allogeneic MSCs together with hematopoietic SC led to less frequent graft-versus-host disease as compared to historical controls, due to the modification of the alloimmune response of MSCs [44]. Additionally, the chronic ischemic tissue itself produces HIF-1α, and contributes to VEGF-mediated responses to chronic injury [45]. Interestingly, no data exist, whether the release of circulating and myocardial tissue HIF-1α is an unspecific host-versus-graft immunomodulatory response or is a specific response of the ischemic myocardium to MSC implantation.

In conclusion, myocardial delivery of MSCs increases the local myocardial expression of HIF-1α in the infarcted area, and elevates the peripheral circulating level of HIF-1α. Intramyocardial delivery of the MSC seems to be a more effective trigger of the release of the angiogenic factor in infarction, because of the probably higher level of SC retention.

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