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The Role of Cytoprotective Cytokines in Cardiac Ischemia/Reperfusion Injury

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The mechanism(s) underlying the beneficial effects of adult mesenchymal stem cells (MSCs) after myocardial infarction (MI) is poorly understood. One possible explanation is the ability of MSCs to secrete cytokines, which modulate cardiomyocyte survival and function. MSCs express at least two cytoprotective cytokines, hepatocyte growth factor (HGF) and stromal cell-derived factor-1 α (CXCL12). The aim of our study was to compare the effects of these two cytokines administered acutely post-MI. We subjected adult male Lewis rats to myocardial ischemia/reperfusion injury. Immediately upon reperfusion, polymers saturated with HGF or CXCL12 were placed onto the infarcted anterior wall and the rats were allowed to recover. Echocardiographic analysis at 4 wk post-MI to assess left ventricular (LV) function revealed that LV ejection fraction was increased in the HGF treated group compared with the phosphate-buffered saline (PBS) control group. Likewise, LV end diastolic dimension was reduced in the HGF treated group compared with the PBS control group. Similarly, invasive hemodynamics at 12 wk showed improved contractility and relaxation in the HGF treated group compared with the PBS control group. In contrast, no significant effect on LV function was seen in the CXCL12 treated group. To determine the potential mechanism for this effect, infarct size (IFS) at 72 h was determined. IFS was decreased 4.2-fold in the HGF treated group compared with the PBS control group. Thus, HGF acutely post-MI using polymer delivery reduces IFS, leading to beneficial effects on post-MI LV remodeling. © 2008 Elsevier Inc. All rights reserved.

Key Words: **adult mesenchymal stem cells; hepatocyte growth factor (HGF); stromal cell-derived factor-1 α (CXCL12); myocardium; ischemia/reperfusion injury.**

INTRODUCTION

In recent years, many investigators have demonstrated the potential of mesenchymal stem cells (MSCs) to be a beneficial therapy after experimental myocardial infarction (MI) by inhibiting pathological remodeling [1]. These cells have shown benefit when delivered acutely after MI [2] and in dilated cardiomyopathy [3]. Although these results confirm the ability of MSCs to beneficially affect remodeling post-MI, the mechanism(s) underlying these effects is poorly understood. It has been proposed that MSCs are capable of differentiating into cardiac myocytes and regenerating cardiac muscle [4]; however, this occurs infrequently [5] despite the beneficial effects on left ventricular (LV) function. Several groups have suggested that MSCs are capable of secreting angiogenic, antiapoptotic, and mitogenic factors [6]. Specifically, one explanation for the beneficial effects is MSCs may secrete cytokines that modulate cardiomyocyte survival and function through an autocrine/paracrine mechanism [7]. In support of this, it has been shown that intramyocardial injection of Akt-overexpressing MSCs reduces ventricular remodeling [8], and more importantly, conditioned medium from cultured MSCs transfected with the serine-threonine kinase Akt/PKB reduces infarct size and apoptotic index in a coronary artery occlusion rat model [9]. Additional sup-

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port of the paracrine hypothesis is provided by data showing that several cytoprotective cytokines (vascular endothelial growth factor [VEGF], fibroblast growth factor-2, hepatocyte growth factor [HGF], insulin-like growth factor-I [IGF-I], and TB4) are significantly up-regulated in the Akt-MSCs [8].

One cytokine capable of activating the PI 3-kinase/Akt pathway in cardiac myocytes is HGF [10]. Originally purified and cloned as a potent mitogen for hepatocytes [11], HGF has pleiotropic, mitogenic, morphogenic, and antiapoptotic properties in various cell types [12]. It is known to play a physiological role as an organotrophic factor for the regeneration and protection of the liver [13], kidney [14], and lung [15]. Previous studies have shown that expression of HGF and its receptor c-Met are transiently up-regulated in the myocardium of developing hearts in mice [16], and HGF plasma levels are markedly elevated in patients with acute myocardial infarction [17]. It has also been shown that HGF gene transfection into myocardium reduces ischemia/reperfusion injury [18]. Moreover, Nakamura *et al.* have recently shown by neutralization of endogenous HGF and intravenous supplementation of recombinant HGF, that endogenous HGF is cardioprotective and exogenous HGF attenuates ischemia/reperfusion injury by directly protecting cardiomyocytes [19].

An additional candidate is stromal cell-derived factor-1 α (CXCL12). CXCL12 and its receptor CXCR4 are necessary for bone marrow retention of hemopoietic stem cells [20], but are also involved in cardiogenesis [21], migration of primordial germ cells [22], and recruitment of endothelial progenitor cells to sites of ischemic tissue [23]. Several studies have suggested that CXCL12 is responsible for stem cell recruitment in injured myocardium [24]. Therefore, it is possible that CXCL12-mediated mobilization of stem cells may represent a viable mechanism for the repair of cardiac tissues.

Adipose-derived mesenchymal stem cells (ASCs) and bone marrow-derived MSCs (BM-SCs) express and secrete both HGF and CXCL12. In light of this, we attempted to determine if direct administration of HGF and CXCL12 was capable of reproducing the effects reported for MSCs; namely, attenuating the damage sustained during ischemia/reperfusion (I/R) injury in the heart. Specifically, the aim of this study was to directly compare the effects of these two cytokines (HGF and CXCL12) expressed by ASCs, when administered acutely post-MI by assessing whether cardiac function improves after MI with increased levels of these cytokines and determining if the improvement in cardiac function is due to a decrease in apoptosis and acute infarct size.

MATERIALS AND METHODS

Isolation and Culture of ASCs and BM-SCs

To obtain rat ASCs, adipose tissue was harvested from the axillary and inguinal fat pads. Tissues were finely minced and resuspended in 20 cc of sterile 0.075% collagenase for every 5 cc of adipose tissue, which was then incubated for 45 to 60 min at 37°C shaking. Samples were then subjected to a regimen of centrifugation, resuspension, and filtration through a gradient of filters (100, 70, and 40 μm), successively. Cells were plated and maintained at subconfluent (~50% to 70% confluence) levels to prevent spontaneous differentiation. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum (FBS), 1% penicillin (10,000 I.U.)-streptomycin (10,000 μg/mL) (Mediatech, Inc., Herndon, VA). All tissue culture reagents and FBS were purchased from Invitrogen (Carlsbad, CA).

Human adipose tissue was obtained from elective liposuction procedures under local anesthesia. The raw lipoaspirate was processed according to established methodologies [25]. Briefly, lipoaspirates were washed extensively with phosphate-buffered saline (PBS) to remove contaminating blood cells, and the extracellular matrix was digested at 37°C for 45 min with 0.075% collagenase I. Enzyme activity was neutralized with DMEM containing 10% FBS, and centrifugation at 1200 rpm for 10 min was done to obtain a high cell density. The pellet was resuspended in DMEM, filtered through a 75-mm nylon mesh to remove cellular debris, centrifuged, and then plated on culture dishes. Human ASCs were cultured in conditions similar to rat ASCs.

BM-SCs were isolated from femurs and tibias of adult rats. The rats were euthanized with an overdose of isoflurane. The tibias and femurs were placed on ice in DMEM. Epiphyses of femurs and tibias were removed, and the marrow was flushed out by using a syringe filled with medium. The bone marrow was then filtered through a 70 μm nylon mesh and plated. Twenty-four hours after plating, supernatant containing nonadherent cells was removed. BM-SCs were then cultured in conditions similar to ASCs.

RNA Analysis

To identify cytokines, which have been reported to have cell survival and/or stem cell homing properties expressed by ASCs, reverse-transcriptase polymerase chain reaction (PCR) for HGF and CXCL12, and their respective receptors c-Met and CXCR4, together with VEGFR1, IG, and IGF1R was performed on cDNA samples prepared from both human and rat adipose tissue using conventional protocols.

To determine the endogenous expression of HGF and c-Met after myocardial infarction, isolation of total RNA was carried out as per the manufacturer's instructions using RNeasy kits (Qiagen, CA). Relative gene expression was measured by a real-time PCR (RT-PCR) system (Applied Biosystems 7700) using 1 ng of template RNA and the QuantiTect SYBR Green RT-PCR kit (Qiagen). Custom primer sequences were designed for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH), HGF, and c-Met mRNA using online software (IDT Technologies, www.idtdna.com). Amplification data were quantified by the comparative threshold cycle method in which relative expression of the target gene is normalized to both GAPDH mRNA expression and target gene expression in noninfarcted hearts (baseline). Forward (F) and reverse (R) primer sequences for the genes were as follows: GAPDH (F): TGCACCACCAACTGCTTA, GAPDH (R): GGATGCAGGGATGATGTTC; HGF (F): ACCTGCAACGGTGAAAGCTACA, HGF (R): AATTTGTGCCGGTGTGGTGTCT; c-Met (F): AATCTCCACTGGCACACTGCAT, c-Met (R): TTTAGCTCGCCGTTCAGCTTCA.

Protein Analysis

To measure cytokine protein expression and secretion, rat ASCs, and BM-SCs were cultured in standard medium (DMEM +10% FBS +1% P/S) until reaching 70% confluence. The culture medium was then replaced with DMEM 3 d afterward, harvesting the cells and medium. Total protein was extracted from the cell pellet using RIPA lysis buffer, and medium using acetone protocol. Protein concentration was determined using the Bradford method. Protein was nor-

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