Activation of host tissue trophic factors through JAK-STAT3 signaling: a mechanism of mesenchymal stem cell-mediated cardiac repair

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Submitted 20 May 2010; accepted in final form 30 August 2010

Shabbir A, Zisa D, Lin H, Mastri M, Roloff G, Suzuki G, Lee T. Activation of host tissue trophic factors through JAK-STAT3 signaling: a mechanism of mesenchymal stem cell-mediated cardiac repair. Am J Physiol Heart Circ Physiol 299: H1428–H1438, 2010. First published September 17, 2010; doi:10.1152/ajpheart.00488.2010.—We recently demonstrated a cardiac therapeutic regimen based on injection of bone marrow mesenchymal stem cells (MSCs) into the skeletal muscle. Although the injected MSCs were trapped in the local musculature, the extracardiac cell delivery approach repaired the failing hamster heart. This finding uncovers a tissue repair mechanism mediated by trophic factors derived from the injected MSCs and local musculature that can be exploited for minimally invasive stem cell therapy. However, the trophic factors involved in cardiac repair and their actions remain largely undefined. We demonstrate here a role of MSC-derived IL-6-type cytokines in cardiac repair through engagement of the skeletal muscle JAK-STAT3 axis. The MSC IL-6-type cytokines activated JAK-STAT3 signaling in cultured C2C12 skeletal myocytes and caused increased expression of the STAT3 target genes hepatocyte growth factor (HGF) and VEGF, which was inhibited by glycoprotein 130 (gp130) blockade. These in vitro findings were corroborated by in vivo studies, showing that the MSC-injected hamstrings exhibited activated JAK-STAT3 signaling and increased growth factor/cytokine production. Elevated host tissue growth factor levels were also detected in quadriceps, liver, and brain, suggesting a possible global trophic effect. Paracrine actions of these host tissue-derived factors activated the endogenous cardiac repair mechanisms in the diseased heart mediated by Akt, ERK, and JAK-STAT3. Administration of the cell-permeable JAK-STAT inhibitor WP1066 abrogated MSC-mediated host tissue growth factor expression and functional improvement. The study illustrates that the host tissue trophic factor network can be activated by MSC-mediated JAK-STAT3 signaling for tissue repair.

IL-6-type cytokine; signal transducer and activator of transcription 3; skeletal muscle
notion, intramuscular administration of VEGF, also a STAT3 target gene, was found to improve ventricular function through similar mechanisms (76, 77). We hypothesize that MSC-derived IL-6-type cytokines activate the skeletal muscle JAK-STAT3 axis, and this upstream signaling event leads to amplification of the host trophic factor network and cardiac repair. The work presented here was initiated to test this hypothesis.

MATERIALS AND METHODS

Animals. F1B (normal) and TO2 (cardiomyopathic) male hamsters (4–5 mo of age) were obtained from Bio Breeders (Watertown, MA). All procedures and protocols conformed to institutional guidelines for the care and use of animals in research, and the study was approved by the University at Buffalo institutional animal care and use committee (IACUC).

Echocardiography. Animals were anesthetized by intraperitoneal injection of xylazine (2 mg/kg) and ketamine (25 mg/kg) and remained semiconscious during the measurement procedure. Multiple M-mode images were obtained from the short-axis view of the left ventricle at the level of the papillary muscles with a GE Vingmed echo machine using a 10-MHz transducer. From this image, left ventricular end-systolic dimension and left ventricular end-diastolic dimension were measured in a blinded manner. These dimensions were measured and averaged from at least two consecutive cardiac cycles.

MSC implantation and drug administration. Porcine MSCs and intramuscular MSC injections were described previously (29, 54, 65). In brief, each TO2 hamster received injections of 4 million MSCs intramuscularly twice per week. The drug was administered by intraperitoneal injection twice a week at a dose of 40 mg/kg as documented (18). The IC$_{50}$ of WP1066 against the malignant glioma U87-MG and U373-MG cells were 5.6 and 3.7 μM, respectively (18). The mean achieved peak plasma concentration following a 40 mg/kg intravenous dose in mice was 4.13 μM, and mean drug half-life was 4.5 h (33).

Cell culture. Porcine MSCs, human MSCs, C2C12 skeletal myocytes, and human embryonic kidney (HEK-293) cells were maintained in DMEM/F-12 supplemented with 10% FBS, 2 mM glutamine, 50 μg/ml gentamicin, and 0.125 μg/ml Fungizone. MSC-CM was prepared by exposing an ~80% confluent MSC culture to a 5% FBS-containing DMEM/F-12 for 2 days. Recombinant mouse IL-6 was from R&D Systems (Minneapolis, MN). Recombinant mouse LIF and human IL-11 were from Millipore (Temecula, CA). For measurement of JAK-STAT3 signaling, C2C12 cells seeded in 6-well plates at a density of 100,000 cells per well were treated with MSC-CM, HEK-293-CM, IL-6, LIF, or IL-11 for 30 min. Cells were then washed twice and lysed in 1% SDS. Whole cell lysates were clarified briefly by centrifugation, and protein concentrations were determined using the Pierce BCA protein assay kit (Pierce Biotechnology, Rockford, IL).

Western blotting. Proteins were resolved by SDS-PAGE and electrophoretically transferred to Immobilon-P membrane, which was incubated with a 1,000-fold diluted primary antibody solution overnight at 4°C. Washed membrane was probed with a horseradish peroxidase (HRP)-conjugated secondary antibody at ~10 ng/ml. Signals were developed using the SuperSignal chemiluminescent substrate from Pierce Biotechnology and imaged by Fuji imager. The following primary antibodies were purchased from Cell Signaling Technology (Danvers, MA): anti-phosphorylated Tyr705 (anti-p-Tyr705) of STAT3 (cat. no. 9145), anti-total STAT3 (cat. no. 9143), anti-p-Tyr1022/1023 of JAK1 (cat. no. 3133), anti-total JAK1 (cat. no. 3332), anti-p-Tyr1007/1008 of JAK2 (cat. no. 3766), anti-total JAK2 (cat. no. 3230), anti-p-Ser473 of Akt (cat. no. 4691), anti-p-Thr202/204 of ERK1/2 (cat. no. 4370), and anti-total ERK1/2 (cat. no. 4695).

Table 1. Primer sequences for qRT-PCR

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<th>Gene</th>
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qRT-PCR, quantitative RT-PCR.
ELISA analysis. Trophic factor concentrations of cell culture media and whole tissue/cell extracts (soluble fraction) were measured using ELISA kits from R&D Systems: IL-6 DuoSet (cat. no. DY686), IL-11 DuoSet (cat. no. DY218), HGF DuoSet (cat. no. DY2207), IGF-II DuoSet (cat. no. DY792), and VEGF DuoSet (cat. nos. DY564 and DY293B). Snap-frozen tissues or cells were homogenized in an ice-cold lysis solution (normal saline supplemented with 0.1% Triton X-100 and 2 mM EDTA). Tissue homogenates were clarified, diluted to 1 mg proteins/ml, and stored at −80°C.

qRT-PCR. RNA extraction from C2C12 skeletal myocytes, MSCs, and hamster tissues were performed using Qiagen RNA isolation kits, and quantitative RT-PCR (qRT-PCR) protocols were as previously described (29, 76). In brief, PCR was performed using the MyiQ machine with the SYBR Green kit (Bio-Rad, Hercules, CA). Amplification conditions after an initial denaturation step for 3 min at 95°C were 45 cycles of 95°C, 10 s, for denaturation, and 55°C, 30 s, for annealing and elongation. Melting curve analysis was performed to check for a single amplicon. MyiQ analysis software was used for determining crossing points. Data were analyzed by the 2−ΔΔCT threshold cycle method. Oligonucleotides were synthesized by Midland Certified Reagent (Midland, TX). Primer sequences are listed in Table 1.

In situ immunostaining. C2C12 myocytes were treated with MSC-CM for 30 min and fixed with 4% paraformaldehyde and permeabilized with 100% methanol. Stained sections were imaged with the Zeiss Axio Imager Z1 epifluorescence microscope. Hamstring cryosections were stained with diluted primary antibodies overnight at 4°C. Sections were further incubated with Alexa 647- and/or Alexa 488-conjugated secondary antibody for 30 min and then mounted in Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA). Stained sections were imaged with an epifluorescence microscope (Zeiss Axio Imager Z1). The myosin heavy chain (MHC) antibody was described previously (25). The β-actin antibody was purchased from Sigma (cat. no. B5186).

Statistical analysis. Data are expressed as means ± SE. Comparisons of parameters between groups were made with unpaired Student’s t-test or ANOVA where applicable. A value of P < 0.05 was considered significant.

RESULTS

MSCs activate JAK-STAT3 signaling in cultured skeletal myocytes. Several IL-6-type cytokines are known to be expressed by MSCs (29, 40, 77). To test the hypothesis that MSC-derived IL-6-type cytokines activate the skeletal muscle JAK-STAT3 axis, we first studied JAK-STAT3 signaling in cultured C2C12 skeletal myocytes in response to MSC-derived...
factors. C2C12 cells were incubated with MSC-CM, and JAK-STAT3 signaling was monitored by Western blot assays of protein phosphorylation. Figure 1A shows that MSC-CM induced phosphorylation of Tyr1022/1023 of JAK1 and Tyr1007/1008 of JAK2. Prominent phosphorylation Tyr705 of STAT3 appeared at 30 min (Fig. 1A), which was further validated by in situ immunostaining of nuclear p-STAT3 (Fig. 1B). Recombinant IL-6, IL-11, and LIF each activated STAT3 phosphorylation as expected (Fig. 1C). In contrast, the conditioned medium prepared from HEK-293 had no detectable effect on STAT3 activation (Fig. 1C). qRT-PCR shows that STAT3 signaling controls a wide array of cellular processes by targeting the expression of many genes such as those involved in cell cycle control (c-myc and cyclin) and encoding trophic factors (HGF, VEGF, and IGF-II). qRT-PCR assays of the injected hamstrings using rodent-specific primers confirm the in vitro data. The MSC-injected hamstrings exhibited a significant increase in STAT3 phosphorylation compared with the saline (HBSS)-injected muscles as shown by Western blotting (Fig. 3A) and in situ immunostaining (Fig. 3B). The p-STAT3-positive nuclei were not detected in the control hamstrings.

**MSC-mediated STAT3 signaling stimulates growth factor/cytokine production in vivo.** Extracardiac MSC injection experiments as described previously (54) were performed to confirm the in vitro data. The MSC-injected hamstrings exhibited a significant increase in STAT3 phosphorylation compared with the saline (HBSS)-injected muscles as shown by Western blotting (Fig. 3A) and in situ immunostaining (Fig. 3B). The p-STAT3-positive nuclei were not detected in the control hamstrings. STAT3 signaling controls a wide array of cellular processes by targeting the expression of many genes such as those involved in cell cycle control (c-myc and cyclin) and encoding trophic factors (HGF, VEGF, and IGF-II). qRT-PCR assays of the injected hamstrings using rodent-specific primers reveal that there was significant induction of the STAT3-regulated genes HGF, VEGF, cyclin D, IL-6, c-myc, and suppressor of cytokine signaling 3 (SOCS3; Fig. 4A). Assays of hamstring homogenates using rodent-specific ELISA kits showed increased amounts of host muscle-derived HGF, VEGF, and IGF-II (Fig. 4B). HGF was ~10 times more abundant than VEGF in the control hamstrings (~0.2 ng HGF/mg vs. ~0.02 ng VEGF/mg soluble proteins). After MSC stimulation, the hamstring HGF concentration increased to ~1 ng/mg, whereas that of VEGF was doubled (Fig. 4B). As illustrated for contracting skeletal muscle (45), some of the muscle-derived growth factors were likely released to the systemic circulation, causing increased circulating levels of HGF and VEGF (see below).

**Paracrine activation of growth factor signaling and expression in the myocardium.** Since the hamstring-injected MSCs were trapped in the local musculature (53), growth factors derived from the MSC-activated hamstrings likely exert their trophic or paracrine actions on the myocardium through multiple signaling pathways, resulting in attenuation of myocardial
apoptosis and promotion of cardiac regeneration (54). Figure 5A shows significant activation of Akt (phosphorylation of Ser473) and ERK1/2 (phosphorylation of Thr202/204) along with enhanced STAT3 signaling in the TO2 hamster heart after MSC therapy. These prosurvival signaling events further caused increased expression of several myocardial growth factors including HGF and VEGF (Fig. 5B), which was also confirmed by ELISA assays of tissue homogenates (Fig. 5C). Taken together, these results illustrate a therapeutically relevant trophic factor signaling cascade initiated by MSC IL-6-type cytokines, which stimulate the skeletal muscle trophic factor network through JAK-STAT3 signaling. Paracrine actions of these host muscle-derived growth factors serve to activate growth factor expression in the diseased heart and mediate myocardial regeneration (54).

Increased growth factor levels in multiple host tissues. We further determined whether the hamstring-injected MSCs, which were trapped in the local musculature (53), might affect
the growth factor levels in other noninjected tissues. Rodent-specific ELISA revealed that liver homogenates contained significantly elevated levels of HGF and VEGF after MSC therapy (Fig. 6A) despite the finding from PCR assays that the intramuscularly injected MSCs could not be detected in the liver (53). A similar paracrine effect was observed for the noninjected skeletal muscle quadriceps (Fig. 6B). Interestingly, a slight but significant increase in HGF content was also detected in the brain after MSC therapy (Fig. 6C). Thus the growth factor trophic mechanism mediated by the MSC-skeletal muscle circuit might have a global physiological effect not restricted to the diseased heart.

Inhibition of JAK-STAT3 signaling abrogates the therapeutic effect of MSCs. To further demonstrate the functional relevance of the proposed signaling cascade, we assessed whether the JAK-STAT3 inhibitor WP1066, which is a cell-permeable analog of AG490 (18), might block the cardiac therapeutic effects of MSCs. Incubation of C2C12 myocytes...
with WP1066 in culture confirmed the STAT3-inhibitory effect of the drug (Fig. 7A). Cardiomyopathic hamsters were divided into 4 groups: WP1066; WP1066 vehicle (PEG); MSC and WP1066; and MSC and WP1066 vehicle (MSC+PEG). The TO2 hamster heart exhibits progressive decline in left ventricular function (35). Myocardial expression of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), which are typically increased in congestive heart failure (37), were increased by ~10- and ~3-fold, respectively, in the 5-mo-old TO2 hamster heart. These changes are associated with dilation of the left ventricular chamber and decreased capillary density, which can be reversed by MSC administration (54). As expected, qRT-PCR assays show that although MSCs attenuated the expression of ANP and BNP, administration of WP1066 blocked this effect of MSCs (Fig. 7B). Furthermore, echocardiography shows that whereas MSCs prevented the progressive decline in left ventricular ejection fraction, WP1066 again abolished this effect of MSCs (Fig. 8). Significantly, these inhibitory effects of WP1066 on heart function were associated with abrogation of MSC-induced HGF and VEGF in the injected hamstrings (Fig. 9A) and in the circulation (Fig. 9B). These studies thus confirm the role of MSC-mediated JAK-STAT3 signaling in amplifying the host tissue trophic factor network.

DISCUSSION

Although it has increasingly been recognized that the trophic actions of MSCs underlie their therapeutic effects in various preclinical and clinical studies (4, 12, 47), the trophic factors responsible for tissue functional improvement remain largely undefined. We demonstrate that MSC IL-6-type cytokines through JAK-STAT3 signaling are involved in the tissue-healing mechanisms. The hallmark of the MSC cardiac repair mechanism lies in the synergistic trophic actions mediated initially by MSC IL-6-type cytokines and subsequently by the myriad of host tissue-derived growth factors, activating the myocardial prosurvival and myogenic pathways orchestrated by Akt, ERK, and JAK-STAT3.

The hamster heart failure model. The δ-sarcoglycan-null strain of TO2 hamster exhibits cardiac dysfunction between 1 and 2 mo of age and develops dilated cardiomyopathy (DCM), which leads to congestive heart failure resembling that seen in many patients (13, 34, 50). The δ-sarcoglycan protein deficiency causes cell membrane fragility and permeability to Ca$^{2+}$, inducing damages to myocytes, smooth muscle cells, and endothelial cells (20, 30, 49, 55). We (35) previously showed that cardiac dysfunction in TO2 hamsters exhibit presymptomatic and symptomatic stages associated with unique biochemical events, and a major pathological hallmark is oxidative stress injury caused by inflammation. Since the hamster DCM model displays successive and uniform phases of pathophysiological changes, it has been used in growth factor and pharmacological therapeutic studies (8, 22, 36, 51). Using this heart failure model, we recently demonstrated cardiac therapeutic effects of extracellular MSCs, which are mediated by increased circulating growth factors, mobilization of bone marrow progenitor cells, attenuation of myocardial cell death and fibrosis, amplification of cardiac progenitor cells, and accelerated cardiomyogenesis and angiogenesis (54, 77).

MSC-mediated JAK-STAT3 signaling in the maintenance of muscle function. JAK-STAT3 signaling is known to promote normal functioning of the skeletal muscle and cardiac muscle systems (15, 26, 27, 63). In particular, altered JAK-STAT3 signaling has been associated with end-stage DCM and myocardial aging (2, 28, 46). We demonstrate a role of MSC IL-6-type cytokines in cardiac repair through engagement of the skeletal muscle JAK-STAT3 axis. The cytokines activate skeletal muscle JAK-STAT3 signaling both in vitro and in vivo with induction of many STAT3 target genes, most notably HGF and VEGF. Functional relevance of this signaling cascade is revealed by attenuated growth factor expression and loss of cardiac therapeutic effect after gp130-JAK-STAT3 blockade. In agreement with our findings here, activation of JAK-STAT3 in skeletal myocytes was found to stimulate HGF and IGF-II expression (70). IL-6 produced by growing myofibers stimulates satellite cell proliferation and skeletal muscle hypertrophy in a STAT3-dependent manner (52), and similar findings have also been presented for LIF (57). Notably, although MSCs were delivered to the hamstrings in our therapeutic trials, the MSC trophic actions further activated STAT3 signaling in the myocardium, which likely plays a significant role in rescuing the failing hamster heart because
Fig. 8. WP1066 blocks MSC-mediated improvement in cardiac function. Cardiac function was determined by blindfolded echocardiography after MSC administration. A: representative M-mode recording for the 4 animal groups at 4 wk. Red arrows were placed to measure left ventricular end-diastolic and -systolic diameters and wall thickness. B: left ventricular ejection fraction (LVEF) at 2 and 4 wk after MSCs. ANOVA was performed to assess statistical significance. *P < 0.05 vs. PEG; †P < 0.05 vs. WP1066; #P < 0.05 vs. preinjection.

Fig. 9. Inhibition of JAK-STAT3 signaling abolishes MSC-induced tissue growth factor levels. Soluble fractions of total hamstring extracts (A) and plasma samples (B) were assayed using rodent-specific HGF and VEGF ELISA kits (n = 4 per group). Tissue growth factor concentrations were expressed as nanograms per milligram soluble proteins. ANOVA was performed to assess statistical significance. *P < 0.05 vs. PEG; †P < 0.05 vs. WP1066; #P < 0.05 vs. MSC+WP1066.
mice harboring a heart-restricted knockout of gp130 or STAT3 develop heart failure (15, 16). At the cellular level, cardiomyocytes with STAT3 deletion produce significantly more TNF-α in response to lipopolysaccharide than normal cardiomyocytes, indicating crucial functions of STAT3 in cardiac resistance to inflammation and acute injury (19). Conversely, cardiac-specific overexpression of STAT3 protects the heart from hypoxia/reoxygenation-induced oxidative stress (39) and promotes myocardial vascular growth (43). Since MSCs abundantly produce IL-6-type cytokines, synergistic actions of these cytokines can efficiently activate the host JAK-STAT3 machinery for tissue repair.

**Amplification of the host trophic factor network for tissue repair.** Among the many STAT3-regulated genes are the growth factors HGF, VEGF, and IGF-II (11, 70, 72), and these trophic factors were induced by MSCs in cultured C2C12 myocytes and after extracardiac MSC delivery. A feature of the growth factor network are cross talk mechanisms that enable induction and amplification of more than one growth factor by another (56, 66, 76). Coordinated induction of muscle HGF and VEGF as observed in our study is noteworthy because combined administrations of HGF and VEGF have been found to result in a much more robust angiogenic response than either growth factor alone (66, 75). Induced HGF and VEGF can further amplify each other through mutual activation (66, 73). This cross-activation mechanism may explain why intramuscular injection of VEGF or HGF alone also repairs the failing hamster heart (24, 76). Similarly, it has been demonstrated that myocardial expression of HGF, IGF, and VEGF were upregulated after intramyocardial stem cell implantation (6). These findings together illustrate the central roles of host tissue trophic factors in regenerative medicine.

Active skeletal muscle can improve mortality in heart patients (62) and prevent cognitive decline in the elderly (31). The extracardiac MSC injection regimen may be compared with exercise therapy, which is known to increase the release of beneficial factors (HGF, IGF, IL-6, VEGF, and NGF) from skeletal muscle and reduce circulating adiponectin levels in heart failure patients (5, 45, 63, 67, 74). The current work demonstrates that increased host tissue trophic factors are also evident in quadriiceps, liver, and brain after the MSC therapy, suggesting a possible global physiological effect of this cell administration approach. Since the production of beneficial trophic factors can be aberrantly downregulated in human heart disease (1, 21), the extracardiac MSC delivery regimen through its engagement and activation of multiple host tissue trophic factors represents a clinically attractive and testable therapeutic strategy.

**MSC-mediated JAK-STAT3 signaling in immunomodulation.** MSCs possess prominent immunomodulatory properties that can be explored for nonautologous or xenogenic stem cell therapy (64). We have shown that human and porcine MSCs can be used for tissue repair in immunocompetent rodents without triggering adverse host tissue inflammation (53, 54, 77). The potent immunomodulatory effects of MSCs appear to nonspecifically target cells of the immune system, and some of the actions appear to be mediated through STAT3 signaling. For instance, MSC-derived IL-6 was found to inhibit the differentiation of bone marrow progenitors into mature dendritic cells (9), thus impairing their stimulatory effect on resting natural killer cells and compromising antigen presentation to T cells. MSC-derived IL-11 and LIF can exert anti-inflammatory actions because these cytokines through STAT3 signaling can inhibit inflammatory cytokine production (14). Indeed, we (53) and others (42, 71) have shown that the trophic actions of MSCs can decrease host production of inflammatory cytokines while inducing the release of anti-inflammatory cytokines. Along this line, we have found that cultured C2C12 myocytes express increased levels of IL-10 and IL-13 (anti-inflammatory cytokines) and decreased levels of TNF-α (inflammatory cytokine) in response to IL-6 (data not shown), suggesting that the MSC/IL-6-type cytokine circuit can potentially modulate the immune system through the paracrine action of muscle cells. Thus it is possible that other cells capable of actively producing multiple IL-6-type cytokines may similarly improve cardiac function of the TO2 hamster heart through host muscle JAK-STAT3 signaling on intramuscular injection.

In summary, we presented a therapeutic mechanism of extracardiac MSC-mediated cardiac repair through engagement of the skeletal muscle JAK-STAT3 axis. The trophic cascade initiated by skeletal muscle JAK-STAT3 signaling increased growth factor levels in multiple tissues, leading to elevated circulating growth factors such as HGF and VEGF. Synergistic actions of these trophic factors further activate the myocardial repair mechanisms orchestrated by the Akt, ERK, and JAK-STAT3 signaling pathways. The therapeutic regimen is minimally invasive, taking advantage of the versatile ability of skeletal muscle to produce and amplify beneficial growth factors and cytokines, most notably HGF and VEGF, in response to multiple MSC IL-6-type cytokines.

**GRANTS**

This study was supported by National Heart, Lung, and Blood Institute Grant HL-84590.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

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