Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement

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ABSTRACT **We previously reported that intramyocardial injection of bone marrow-derived mesenchymal stem cells overexpressing Akt (Akt-MSCs) inhibits ventricular remodeling and restores cardiac function measured 2 wk after myocardial infarction. Here, we report that the functional improvement occurs in < 72 h. This early remarkable effect cannot be readily attributed to myocardial regeneration from the donor cells. Thus, we hypothesized that paracrine actions exerted by the cells through the release of soluble factors might be important mechanisms of tissue repair and functional improvement after injection of the Akt-MSCs. Indeed, in the current study we demonstrate that conditioned medium from hypoxic Akt-MSCs markedly inhibits hypoxia-induced apoptosis and triggers vigorous spontaneous contraction of adult rat cardiomyocytes in vitro. When injected into infarcted hearts, the Akt-MSC conditioned medium significantly limits infarct size and improves ventricular function relative to controls. Support to the paracrine hypothesis is provided by data showing that several genes, coding for factors (VEGF, FGF-2, HGF, IGF-I, and TB4) that are potential mediators of the effects exerted by the Akt-MSC conditioned medium, are significantly up-regulated in the Akt-MSCs, particularly in response to hypoxia. Taken together, our data support Akt-MSC-mediated paracrine mechanisms of myocardial protection and functional improvement.—Gnecchi, M., He, H., Noiseux, N., Liang, O. D., Zhang, L., Morello, F., Mu, H., Melo, L. G., Pratt, R. E., Ingwall, J. S., Dzau, V. J. Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. *FASEB J.* 20, 661–669 (2006)**

Key Words: cell therapy · cytoprotection · inotropism · myocardial infarction · secreted factors

Different types of bone marrow-derived adult stem cells have been proposed as a potential source for cell therapy of acute myocardial infarction (MI) (1–5). Given their multipotency, low immunogenicity, amenability to ex vivo expansion, and genetic modification, autologous bone marrow-derived mesenchymal stem cells (MSCs) are particularly suitable for this purpose (6–14). Several groups have reported that intramyocardial administration of MSCs reduces postinfarction ventricular remodeling and in some cases improves left ventricular function (15–19). However, the mechanisms underlying these therapeutic effects have not been clearly defined. Although in situ differentiation of the MSCs into cardiomyocytes and other cell types constituting the cardiac tissue has been proposed by some groups (15–19), there is much debate over the frequency of this phenomenon (14). The low periimplantation viability of the injected cells may limit the capacity of this mechanism to result in meaningful cardiac regeneration (14).

We previously reported that the genetic modification of MSCs with the prosurvival gene Akt1 (Akt-MSCs) increases the post-transplantation viability of these cells and enhances their therapeutic efficacy. In fact, intramyocardial injection of Akt-MSCs leads to the prevention of ventricular remodeling and to the restoration of cardiac function when measured 2 wk after the MI (20). In the current study, we first evaluated whether some effect occurred before 2 wk to gain insight into the potential mechanisms underlying the therapeutic action of the Akt-MSCs. Remarkably, with Akt-MSC therapy, the restoration of ventricular function was fully achieved in < 72 h. A recovery occurring so early cannot be attributed to cardiac regeneration from the donor cells. Thus, we reasoned that it might be achieved through myocardial protection and/or enhanced ventricular function. We hypothesized that Akt-MSCs exert beneficial paracrine actions on the ischemic cardiomyocytes by releasing biologically active

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factors. To prove our hypothesis, we tested the cytoprotective effect of conditioned medium from cultured Akt-MSCs on isolated adult rat ventricular cardiomyocytes (ARVCs) exposed to hypoxia in vitro and on rat hearts after experimental MI in vivo. We assessed the contraction characteristics of ARVCs exposed to hypoxia in the presence of conditioned medium. Most importantly, we determined the effect of the conditioned medium on ventricular performance in infarcted rat hearts. Finally, we tested whether the overexpression of Akt up-regulates the expression of candidate paracrine factors with known cytoprotective and/or inotropic properties.

Our current results provide evidence that Akt-MSCs secrete paracrine-acting agents with cardio-protective and/or inotropic properties that can rescue ischemic cardiomyocytes and improve ventricular function. This finding may represent an important breakthrough in understanding stem cell action in tissue protection and repair and in developing effective molecular therapeutics.

MATERIALS AND METHODS

Purification and retroviral transduction of mesenchymal stem cells

MSCs were isolated from the bone marrow of adult Sprague-Dawley male rats (Harlan World Headquarters, Indianapolis, IN, USA) and expanded according to reported protocols (20). The MSCs were fed α-minimum essential medium (α-MEM; from Gibco, Grand Island, NY, USA) and 10% fetal bovine serum. First-passage cells were transduced with a retroviral vector encoding the gene green fluorescent protein (GFP-MSCs) or with a bicistronic vector expressing both the genes GFP and Akt1 (Akt-MSCs). Transduction efficiency was assessed by fluorescence-activated cell sorting (FACS) analysis (Becton Dickinson FACS Vantage, Medford, MA, USA).

Myocardial infarction and cell injection models

Ligation of the left coronary artery (LCA) was performed in 6-wk-old Sprague-Dawley female rats (Harlan World Headquarters, Indianapolis, IN, USA) as described (21). Briefly, animals were anesthetized using a combination of xylazine (10 mg/kg) and ketamine (80 mg/kg) injected intraperitoneum, and a left thoracotomy was performed under artificial ventilation with air. The heart was accessed through the fifth intercostal space. The pericardial sac was cut and the heart exteriorized through the space. The LCA was legated with a silk suture approximately midway between the left atrium and the apex of the heart and EKG was recorded to confirm the presence of infarction. In the sham animals the ligature was left loose and no injection was performed. Before surgery, animals were randomized into four groups: sham-operated animals, control animals that received phosphate-buffered saline (PBS) injection, and cell-treated animals that received either GFP-MSCs or Akt-MSCs. Accordingly, 1 h later a total of 5×10^6 fourth passage GFP-MSCs or Akt-MSCs suspended in PBS or an equivalent volume of PBS alone was injected in five different sites at the infarct border zone. The investigators responsible for surgery and cell injection were blinded to the treatment groups. All procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act and approved by the Harvard Standing Committee on Animals.

Measurement of infarct size and immunohistochemical analysis

Infarct size at 72 h was analyzed with planar morphometry in triphenyltetrazolium chloride (TTC) (Sigma Chemicals, St. Louis, MO, USA) stained sections and expressed as a ratio of the left ventricular area. Each heart was cut into five biventricular sections of similar thickness, which were incubated in a 1% TTC solution (diluted in PBS; pH 7.4) at 37°C for 5 min and fixed for 12 h in 10% phosphate-buffered formalin. Both sides of each slice were photographed with a digital camera (Nikon Coolpix 4500) connected to a stereomicroscope (Nikon SMZ 1500). The boundary of the unstained area (infarcted tissue) was traced in a blinded fashion and quantified with dedicated software (ImageJ from NIH). The sections were then repeatedly washed with PBS, processed, and embedded in paraffin for hematoxylin-eosin (H&E) staining and histopathological analysis of the infarct.

Immunohistochemical analysis was performed on paraffin-embedded sections. To detect the injected cells we used a primary antibody anti-GFP (from Rockland, Gilbertsville, PA, USA) and an appropriate secondary antibody TRITC-conjugated (from Jackson Laboratories, Bar Harbor, ME, USA). To verify whether the injected cells co-expressed cardiac markers, we stained the same sections with a primary antibody anti-α-sarcomeric actin (α-SA; from Sigma) in combination with a specific secondary antibody FITC-conjugated (from Jackson Laboratories).

Measurement of cardiac function

Cardiac function was analyzed 72 h after surgery using a Langendorff preparation as described previously in detail (21). Briefly, the heart was rapidly excised and immersed in ice-cold buffer. The aorta was dissected free and mounted onto a cannula attached to a perfusion apparatus. Retrograde perfusion of the heart with phosphate-free Krebs-Henseleit buffer was started in the Langendorff mode at a constant temperature of 37°C and a constant coronary perfusion pressure of 100 mmHg. Equilibrating the buffer with 95% O_2, 5% CO_2 yielded a pH of 7.4. For measurement of cardiac performance, a water-filled Latex balloon was inserted into the left ventricle through an incision in the left atrial appendage via the mitral valve. The balloon was connected to a Statham P23Db pressure transducer (Gould Instruments, Glen Burnie, MD, USA) with a small-bore polyethylene tubing for continuous measurement of left ventricular pressure and heart rate on a dedicated on-line system (Mac Lab AD Instruments, Milford, MA, USA). We measured the left ventricular systolic pressure (LVSP) as well as rates of tension (+dP/dt) and of relaxation (−dP/dt) development. The investigators responsible for infarct size determination, isolated heart experiments, and data analysis were blinded to the treatment groups.

In vitro experiments with the conditioned medium

Conditioned medium was generated as follows: 90% confluent fourth passage GFP- or Akt-MSCs were fed with serum-free α-MEM and incubated for 12 h under normoxia (N-M) or hypoxia (H-M). The medium was then collected and used for in vitro experiments. Hypoxic conditions were created by incubating the cells at 37°C inside an airtight Plexiglas chamber (Billups Rothenberg, Del Mar, CA, USA) with an atmosphere of 5% CO_2/95% N_2. The oxygen level in the

chamber was ~ 0.5% (oxygen analyzer MAXO$_2$ from Maxtec, Salt Lake City, UT, USA).

ARVCs were isolated from 6-wk-old female Sprague-Dawley rats by enzymatic dissociation according to a standard protocol reported previously (22). Cells were seeded in multi-well plates (Becton Dickinson, Franklin Lakes, NJ, USA) precoated with laminin (1 μg/cm^2) and left overnight in standard growth medium (M199; from Gibco) (22). One day later, the M199 medium was replaced with serum-free α-MEM (control medium; CTR-M) or conditioned medium from either GFP-MSCs or Akt-MSCs. The ARVCs were then placed in the hypoxic chamber. The viability of the ARCMs was evaluated on the basis of their morphology using a phase contrast microscope, and rod-shaped cardiomyocytes were considered viable. The number of viable ARVCs grown in normal conditions was considered as baseline. Caspase 3 was determined using a fluorimetric assay kit (Sigma) in accordance with the manufacturer's recommendations.

In vivo experiments with the concentrated conditioned medium

Concentrated conditioned medium was generated as follows: fourth passage GFP-MSCs or Akt-MSCs were fed with serum-free α-MEM and left inside the hypoxic chamber for 12 h. The medium was then collected and cells were counted for normalization purposes: for each animal we used medium generated by 5×10^6 cells. After removing the cell debris, the supernatant was transferred into dedicated ultrafiltration tubes (Amicon Ultra-PL 5 from Millipore, Bedford, MA, USA), concentrated (cM) and desalted according to the manufacturer's protocol. Control medium (CTR-cM) was generated in the same way except there were no cells in the plate.

A total volume of 600 μL of cM was injected in five different sites at the infarct border zone 30 min after coronary ligation. Infarct size was assessed by TTC staining as described above. Endothelial cells were stained with an antibody anti-leptin I and microvessels, defined as any endothelial cell or group of endothelial cells not surrounded by other cell types, quantified at the infarct border zone as total number per high-power field. Basal cardiac function was determined using Langendorff preparation (21). After assessing the baseline function, a solution of 300 nM dobutamine was infused through a side tube by a digital infusion pump (Cole-Parmer Instrument Company, Vernon Hills, IL, USA) at 2% of the coronary flow rate for 15 min and the ventricular performance was recorded.

RNA analysis

Total RNA was extracted from the MSCs with affinity resin columns (Qiagen, Chatsworth, CA, USA). First strand cDNA was prepared using oligo-dT priming and 100 ng per reaction were subsequently used as a template for quantitative RT-PCR (qRT-PCR). qRT-PCR was performed with Taqman® technology on a 7700 Applied Biosystems sequence detection system with Assays-on-Demand™ primer-probe sets (Applied Biosystems, Foster City, CA, USA). mRNA levels were expressed relative to an endogenous control (18S RNA) and the fold increase in the Akt-MSCs vs. control GFP-MSCs was determined.

Statistics

All results are presented as mean plus or minus standard error and were analyzed with a 1- or 2-way ANOVA post hoc Bonferroni all pairwise multiple comparison test. For qRT-PCR data we used a post hoc Bonferroni multiple comparison of all groups vs. GFP-MSCs at baseline normoxia. Probability (P) values < 0.05 were considered statistically significant.

RESULTS

Intramyocardial injection of Akt-MSCs limits infarct size and improves ventricular function after acute MI as early as 72 h

FACS analysis of the MSCs after viral infection showed a transduction efficiency of ~90% for both the GFP-MSCs and the Akt-MSCs (data not depicted).

The effect of Akt-MSCs on myocardial injury 72 h after infarction was evaluated by TTC and H&E staining (**Fig. 1**). Mean infarct size in the PBS control animals was 34 ± 4% of the left ventricle (LV) (Fig. 1*A*). Injection of GFP-MSCs had a modest protective effect, limiting the size of the infarct to 29% ± 3% of the LV (P=NS) (Fig. 1*B*). In contrast, injection of Akt-MSCs significantly limited infarct size to 13% ± 4 of the LV ($P<0.05$ vs. both the PBS and the GFP-MSC group) (Fig. 1*C*). Compared with the PBS group, this translated into a relative reduction in infarct size of 15% in the GFP and 62% in the Akt group. These results were further confirmed by histopathological examination of H&E-stained sections (Fig. 1*D–F*).

We then evaluated the ventricular function in isolated hearts using a Langendorff preparation, which allowed us to measure the contractile performance independent of loading conditions and neurohormonal factors. Left ventricular function measured at 72 h is shown in **Fig. 2**. The LVSP in the PBS injected hearts was 36% lower than in the sham-operated control hearts ($P<0.05$) (Fig. 2*A*). The LVSP in the GFP-

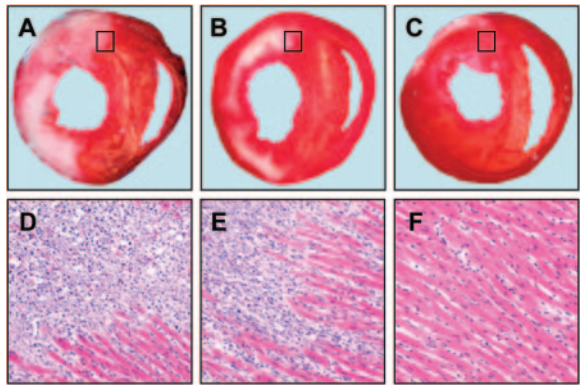

Figure 1. Effect of the MSCs on infarct size and inflammatory response. (*A, D*) The LCA ligation in the PBS control group resulted in an infarct equivalent in size to a third of the entire left ventricular area as calculated by TTC staining (*A*) and a massive infiltration of inflammatory cells as documented by H&E staining (*D*). *B, E)* After injection of GFP-MSCs, infarct size (*B*) and inflammatory response (*E*) were reduced but not significantly. *C, F)* Treatment with Akt-MSCs remarkably limited the infarct size (*C*), as well as the inflammatory response (*F*).

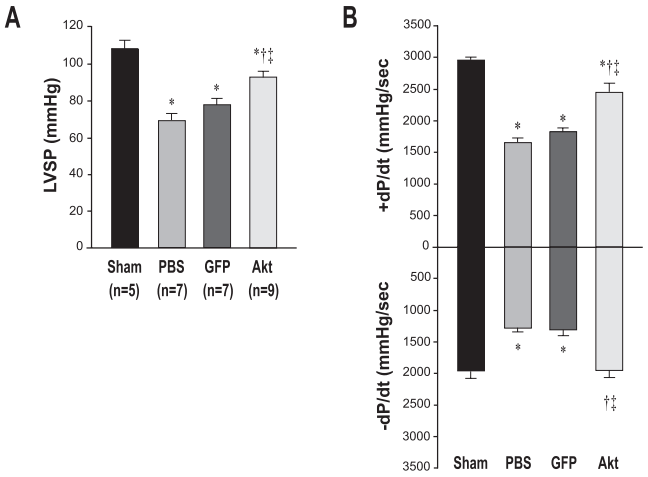

Figure 2. Postmyocardial infarction ex vivo cardiac function after injection of MSCs. *A)* At 72 h after acute MI, LVSP was reduced in the PBS control animals and was slightly but not significantly increased after injection of GFP-MSCs; in the Akt-MSC group, LVSP was significantly improved compared with the PBS and GFP groups. *B)* The LV ±dP/dt deteriorated both in the PBS and GFP group but not in Akt-MSC-treated animals. *$P < 0.05$ vs. sham; †$P < 0.05$ vs. PBS, ‡$P < 0.05$ vs. GFP-MSCs.

MSC-treated animals was similar to the PBS group, while the injection of Akt-MSCs resulted in significantly higher LVSP than in both the PBS and the GFP-MSC group ($P<0.05$) (Fig. 2*A*). In addition, the +dP/dt and −dP/dt followed the same pattern (Fig. 1*B*). The body, heart, and LV weights did not differ among groups (data not shown).

To test whether this marked morphological and functional improvement was due to cardiac regeneration from the donor cells, we performed double staining for GFP and α-SA on paraffin-embedded sections. No GFP-positive cells were detected in the PBS control group (**Fig. 3*A***). On the contrary, we were able to identify the injected MSC, both in the GFP-MSC-treated hearts (Fig. 3*B, C*). However, in both groups none of the MSCs present in the infarcted area co-expressed GFP and α-SA at 72 h (Fig. 3*B, C*). Therefore, the protective effect of injected MSCs cannot be explained by cardiac regeneration from the donor cells.

Akt-MSC conditioned medium protects isolated cardiomyocytes exposed to hypoxia in vitro and limits myocardial infarct size in vivo

Thus, we hypothesized that the Akt-MSCs might release cytoprotective factors able to prevent cardiomyocyte loss. To test our hypothesis, we first studied the effects of conditioned medium from cultured MSCs on the viability of ARVCs subjected to hypoxia in vitro. The ARVCs were exposed to hypoxia for 24 h in the presence of CTR-M, normoxic conditioned medium (N-M), or hypoxic conditioned medium (H-M) from GFP- or Akt-MSCs. ARVCs maintained in CTR-M under normoxic conditions for 24 h (baseline) were mostly viable and exhibited their typical rod-shaped appearance (**Fig. 4*A, G***). Exposure of ARVCs to 24 h of hypoxia in CTR-M resulted in 82% reduction in the number of rod-shaped cells ($P<0.05$) (Fig. 4*B, G*). It is known that the transition of ARVCs from the rod-shaped to the rounded morphology coincides with ultrastructural alterations typical of either necrotic or apoptotic cell death (23). Exposure to the GFP-MSC N-M did not significantly affect the number of rod-shaped ARVCs (Fig. 4*C, G*). In contrast, the Akt-MSC N-M led to a statistically significant increase in the number of rod-shaped ARVCs compared with the CTR-M (+67%; $P<0.05$) (Fig. 4*D, G*). The results obtained in the presence of conditioned medium from MSCs exposed to hypoxia were the most remarkable. Indeed, the GFP-MSC H-M increased by 89% and 53% the number of rod-shaped ARVCs compared with the CTR-M and the GFP-MSC N-M, respectively ($P<0.05$) (Fig. 4*E, G*). Significantly greater protection was achieved with the Akt-MSC H-M (Fig. 4*F, G*): the rod-shaped ARVCs were 3.8-fold more numerous than in the presence of the CTR-M ($P<0.05$) and 2.2-fold more ($P<0.05$) compared with the ARVCs in the Akt-MSC N-M. Finally, compared with the GFP-MSC H-M, the Akt-MSC H-M increased the number of rod-shaped ARVCs by 2.0-fold ($P<0.05$) (Fig. 4*F, G*).

To strengthen these morphological observations and further investigate the protective effect of the conditioned medium, we determined the caspase 3 activity in

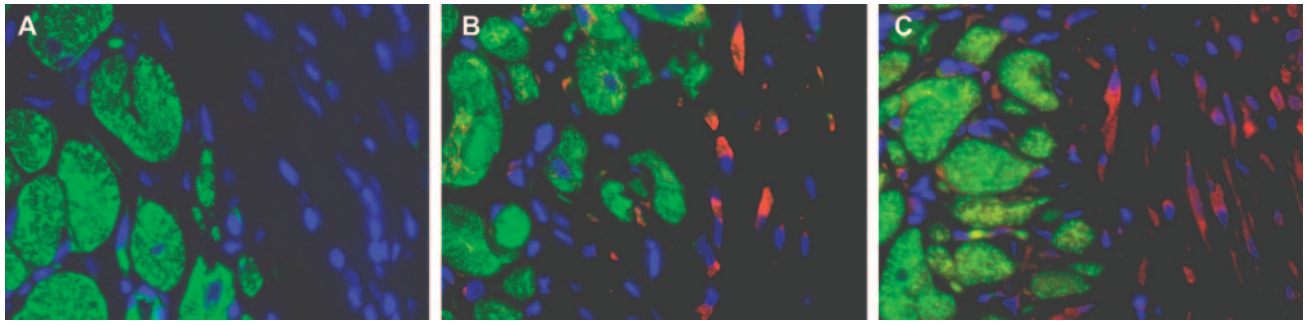

Figure 3. Lack of myogenesis from the donor cells 72 h after MI. *A)* No GFP-positive signal was detected in the PBS control group. *B)* The GFP-MSCs positively stained for GFP (red fluorescent signal) but did not express the specific cardiac marker α-SA (identified by the green fluorescent signal). *C)* Compared with GFP-MSCs, a higher number of Akt-MSCs (in red) engrafted in the scar tissue but none of them co-express α-SA (in green).

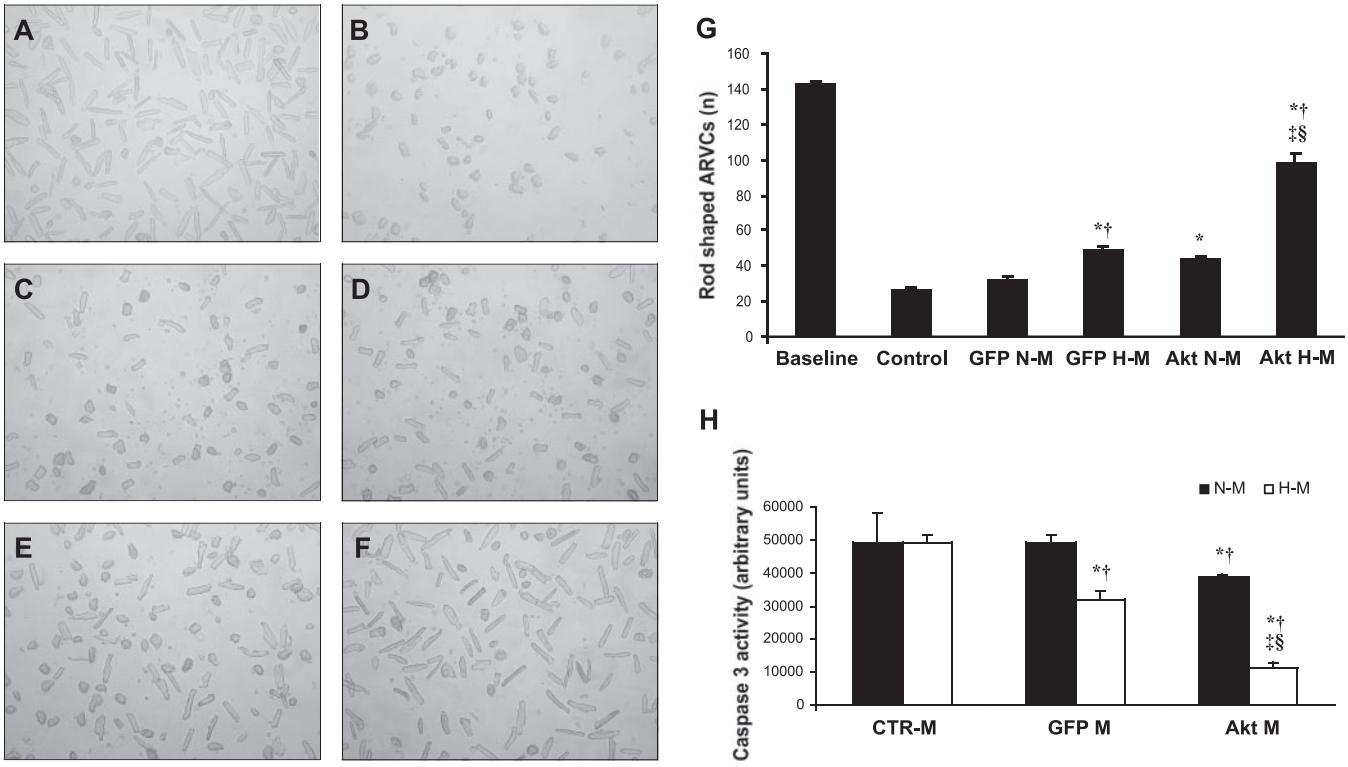

Figure 4. Effect of the MSC conditioned medium on viability and apoptosis of ARVCs exposed to hypoxia. *A)* Representative microphotographs of ARVCs grown in CTR-M under normoxic conditions for 24 h (considered as baseline). *B–F)* ARVCs after 24 h of hypoxia in CTR-M (*B*), GFP-MSC N-M (*C*), Akt-MSC N-M (*D*), GFP-MSC H-M (*E*), or Akt-MSC H-M (*F*). *G)* The bar graph summarizes the results of 3 independent experiments where ARVCs were exposed to hypoxia for 24 h in the presence of CTR-M or different conditioned medium (n=6 fields counted in 3 different wells for each condition). Statistics: *$P < 0.05$ vs. control; †$P < 0.05$ vs. GFP N-M; ‡$P < 0.05$ vs. GFP H-M; §$P < 0.05$ vs. Akt N-M. *H)* Caspase 3 activity of ARVCs after 24 h of hypoxia in the presence of N-M (black bars) or H-M (white bars). Statistics: *$P < 0.05$ vs. normoxic and hypoxic CTR-M; †$P < 0.05$ vs. normoxic GFP-M; ‡$P < 0.05$ vs. hypoxic GFP-M; §$P < 0.05$ vs. normoxic Akt-M.

ARVCs under the same conditions. The GFP-MSC N-M had no significant effect on caspase 3 activity (Fig. 4*H*). In contrast, Akt-MSC N-M significantly reduced caspase 3 activity by 21% compared with the CTR-M (P<0.05) (Fig. 4*H*). Both the GFP-MSC H-M and Akt-MSC H-M significantly decreased the caspase 3 activity (Fig. 4*H*) but the Akt-MSC H-M had a more remarkable effect, reducing caspase activity by 66% and 78% compared with the GFP H-M (P<0.05) and the CTR-M (P<0.05), respectively.

To examine the in vivo relevance of our in vitro findings, we studied the direct effects of the conditioned medium on infarct size in infarcted rat hearts. On the basis of the in vitro results and to streamline the design of the in vivo experiments, we elected to use medium only from MSCs exposed to hypoxia. Concentrated conditioned medium was injected at the infarct border zone 30 min after LCA occlusion. Hearts were isolated 72 h later to determine the infarct size by TTC staining (**Fig. 5*A–C***). The infarct size in the CTR-cM-treated hearts was 33 ± 5% of the left ventricular area (Fig. 5*A*). Injection of GFP-cM slightly limited the infarct to 29 ± 4% (P=NS) of the LV (Fig. 5*B*). Injection of concentrated conditioned medium from Akt-MSCs limited the infarct size to 15 ± 4% (P<0.05) of the LV (Fig. 5*C*).

We also quantified capillary density to examine the possible contribution of neoangiogenesis to the myocardial repair process. The number of microvessels per high-power field present at the infarct border zone was not statistically different among the groups

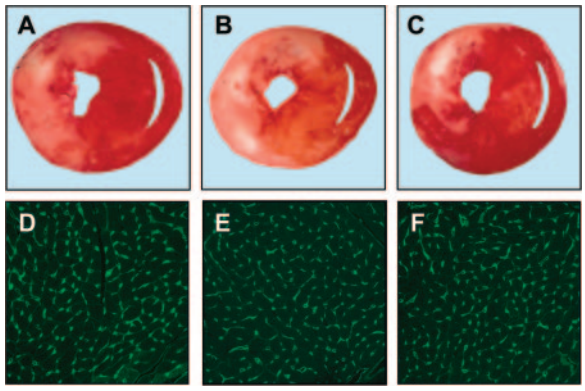

Figure 5. Effect of the concentrated conditioned medium on infarct size and angiogenesis. *A–C)* Infarct size dimensions in the CTR-cM (*A*), GFP-cM (*B*), and Akt-cM (*C*) group, respectively were very similar to those observed in the cell injection counterpart experiment. *D–F)* No differences were observed among the CTR-cM (*D*), GFP-cM (*E*), and Akt-cM (*F*) group in terms of microvessel density quantified at the infarct border zone.

(CTR-cM: 161±19; GFP-cM: 157±20; AKT-cM: 179±23; P=NS) (Fig. 5*D–F*).

Akt-MSC conditioned medium induces spontaneous contraction of ARVCs under prolonged hypoxia in vitro and restores ventricular function after infarction in vivo

We then examined the behavior of ARVCs in real time after 48 h of incubation in the hypoxic chamber. In the presence of CTR-M, very few ARVCs were attached to the plate, and almost all were rounded-up and isolated. Less than 5% of the cells showed spontaneous contractile activity that was slow and irregular (Supplementary information, movie 1). In the presence of GFP-MSC H-M, 28% more ARVCs were attached to the plate ($P<0.05$); the majority were rounded up, and their spontaneous contractility was slow and irregular. However, the number of ARVCs contracting in the presence of GFP-MSC H-M was higher than in the presence of CTR-M, ~ 10–15% of the total number of cells left (Supplementary information, movie 2). In striking contrast, significantly more cells were still attached to the plate in the presence of Akt-MSCs H-M (+3.8-fold compared with the CTR-M; $P<0.05$). The majority of the ARVCs was rounded up and attached to each other. Most importantly, 60–65% of them were spontaneously and strongly beating. In some cases, the contraction of adjacent cells was synchronized and almost simulated an early syncytium (Supplementary information, movie 3).

To test whether factors secreted by the Akt-MSCs alone can rescue the cardiac function and obtain further evidence in vivo that a main mechanism of action of the Akt-MSCs might be through the release of such factors, we assessed the left ventricular function in infarcted rat hearts injected with concentrated conditioned medium. At 72 h after the injection, the LVSP in the CTR-cM group was 72 ± 1.1 mmHg compared with 108 ± 2 mmHg in the sham group, corresponding to a 34% reduction ($P<0.05$) (**Fig. 6*A***). The LVSP in the GFP-cM-treated hearts was 75 ± 2 mmHg, and did not significantly differ from the LVSP recorded in hearts treated with CTR-cM (Fig. 6*A*). In contrast, injection of Akt-cM resulted in a LVSP of 96 ± 2 mmHg, a value 25% and 22% higher than in the CTR-cM ($P<0.05$) and GFP-cM ($P<0.05$) group, respectively (Fig. 6*A*). The LVSP in the Akt-cM group was only 11% lower than in the sham animals. Finally, indices of systolic and diastolic function ±dP/dt were significantly reduced in both the CTR-cM ($P<0.05$) and the GFP-cM ($P<0.05$) group, whereas injection of Akt-cM improved these parameters compared both with those in the CTR-cM ($P<0.05$) and GFP-cM ($P<0.05$) -treated animals. The −dP/dt was completely normalized in the Akt-cM group (Fig. 6*B*). All the parameters of ventricular function in both treatment groups were very similar between cell and conditioned medium injection experiments.

We also examined the response of the treated hearts

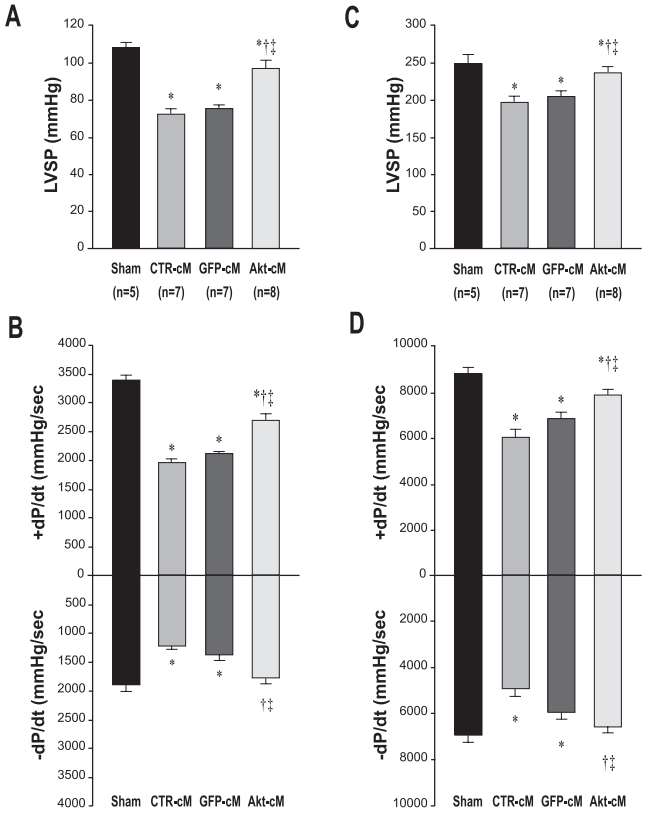

Figure 6. Postmyocardial infarction ex vivo cardiac function after injection of concentrated conditioned medium. *A)* At 72 h after the MI, the LVSP was reduced in animals injected with the CTR-cM or the GFP-cM whereas the injection of Akt-cM resulted in improved LVSP. *B)* Similar results were obtained in terms of +dP/dt; the −dP/dt was *n*ormalized in the hearts injected with Akt-cM. *C, D)* The Akt-cM-treated hearts exhibited significantly greater inotropic response to dobutamine than the other control groups. *$P < 0.05$ vs. sham; †$P < 0.05$ vs. CTR-cM, ‡$P < 0.05$ vs. GFP-cM.

to an inotropic stimulus such as dobutamine (Fig. 6*C, D*). We analyzed the functional parameters recorded during the first 3 and the last 5 min of the total 15 min of dobutamine infusion. During the first 3 min, in both the control and treated animals we observed increases in the LVSP and ±dP/dt compared with baseline. However, the absolute values of all the functional parameters were significantly higher in the Akt-cM-treated hearts compared with the CTR-cM and the GFP-cM group ($P<0.05$) (Fig. 6*B, C*). We observed a similar trend during the last 5 min of dobutamine infusion (data not shown). Thus, the Akt-cM-treated hearts have a better ventricular function not only in basal conditions but also in response to inotropic stimulation.

Akt-MSCs are capable of up-regulating the gene expression of secreted factors

To verify whether Akt overexpression up-regulates the expression of secreted factors, we tested by qRT-PCR some candidate genes encoding for molecules known

to be released by the MSCs. The RNA was extracted from both GFP-MSCs and Akt-MSCs after 4 h of exposure to normoxia or hypoxia. qRT-PCR was performed for vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-I), and thymosin β4 (TB4). Results were expressed as relative fold change compared with the GFP-MSCs grown under normoxia. Our data showed that all of the above genes were significantly up-regulated in the Akt-MSCs at baseline normoxia ($P<0.05$ vs. GFP-MSCs under normoxia) and increased further after exposure to hypoxia ($P<0.05$ vs. Akt-MSCs under normoxia) (**Fig. 7**). After exposure to hypoxia, the GFP-MSCs also up-regulated VEGF, bFGF, HGF, and TB4 ($P<0.05$) even though significantly less than the Akt-MSCs ($P<0.05$ vs. Akt-MSCs under hypoxia) (Fig. 7).

DISCUSSION

We previously showed that the intramyocardial injection of bone marrow-derived MSCs overexpressing Akt is a superior strategy for rescuing tissue damage caused by acute infarction compared with the administration of control MSCs (20). Here, we provide new evidence on the mechanisms of action of the Akt-MSCs. We demonstrate that, especially when exposed to hypoxia, Akt-MSCs release factors that exert beneficial effects on isolated cardiomyocytes in vitro. We also show that intramyocardial injection of conditioned medium containing these putative substances significantly improves left ventricular function in vivo as early as 72 h. These new data strengthen our recent observation on the cytoprotective properties of the Akt-MSCs (24) and strongly support the concept that the effects observed after intramyocardial injection of Akt-MSCs into infarcted hearts are to a great extent attributable to paracrine protection and functional recovery of ischemic myocardium.

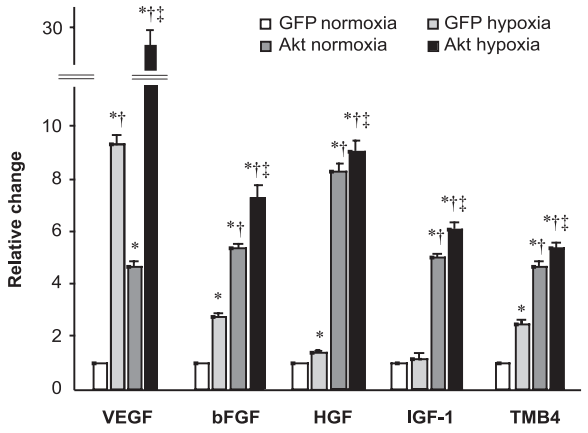

Figure 7. Effect of hypoxia on the MSC expression of selective candidate paracrine factors. Transcript levels of selective soluble factors were significantly up-regulated in the Akt-MSCs compared with control GFP-MSCs under normoxia and further increased after exposure to hypoxia for 4 h. *$P < 0.05$ vs. GFP-MSC normoxia; †$P < 0.05$ vs. GFP hypoxia; ‡$P < 0.05$ vs. Akt-MSC normoxia.

This paradigm is supported by the observation that infarct size limitation and functional recovery occurred in <72 h after Akt-MSCs transplantation into infarcted hearts without evidence of meaningful myogenesis from the donor cells. Indeed, we could not detect cells coexpressing GFP and specific cardiac markers in the infarcted zone. Only a few double positive cells were identified at the infarct border zone. These cells did not differ in size and shape from the surrounding cardiomyocytes, strongly suggesting they were the product of cell fusion. Although in the present study we did not address the role of cell fusion in early myocardial recovery, our recent data from a separate study show that the frequency of fusion events at the infarct border zone after Akt-MSC injection is too low to explain the reduction of myocardial injury and the improvement of cardiac function (unpublished data). Partial replacement of the infarcted tissue with new cardiomyocytes derived from circulating or resident stem cells attracted by factor(s) released by the Akt-MSCs cannot be excluded. However, even though the presence of regeneration from endogenous cells could partially explain the reduction of the infarct size, this probably would not account for the functional data observed. In fact, it seems unlikely that the maturation of these cells to fully competent cardiomyocytes can occur in <72 h (25, 26). On the contrary, this mechanism might be very important in the long-term remodeling process, and additional experiments are needed to determine whether endogenous regeneration is indeed present in our model.

Support for our paracrine hypothesis is provided by results obtained with the conditioned medium. We showed first that conditioned medium from hypoxic MSCs exerts a striking protective effect on isolated ARVCs exposed to hypoxia. We then injected concentrated conditioned medium from hypoxic MSCs into infarcted hearts, obtaining remarkable protection with the Akt-MSC conditioned medium in vivo. A rapid occurrence of neoangiogenesis does not seem likely to be a main mechanism involved, since no significant increase in microvessel density was present in the treatment groups 72 h after injection of the Akt-MSC conditioned medium.

With regard to the early functional improvement of acutely infarcted hearts after injection of Akt-MSCs or their conditioned medium, the effect may be the consequence of myocardial protection leading to infarct size limitation and/or the direct action of inotropic factors released by the cells. As for the former mechanism, it has been well documented that therapies able to limit myocardial infarct size can prevent ventricular dysfunction (27). In support of the latter, we observed that, in the presence of the conditioned medium from hypoxic Akt-MSCs, there was a marked increase in spontaneous contractile activity in isolated ARVCs exposed to prolonged hypoxia. Such contractile activity was very different from the typical spontaneous contractility observed when the ARVCs are maintained in standard growth

conditions. In this case, the vigorous and synchronized contraction observed may suggest the presence of inotropic factors able to positively influence the contractile properties of the cardiomyocytes in the conditioned medium. However, the relative contribution of the two proposed mechanisms cannot be determined by our current experiments.

The hypothesis of paracrine actions mediated by the Akt-MSCs is also supported by our data showing that the Akt-MSCs are capable of up-regulating the expression of several candidate mediators such as VEGF, FGF-2, HGF, IGF-I, and TB4 at baseline and, more significantly, after exposure to hypoxia. These candidates were randomly selected among others because they are known to be secreted by the MSCs and to exert pro-angiogenetic, cardioprotective, and inotropic actions (28–31). Even though this experiment does not provide mechanistic evidence that these particular factors are in fact responsible for the observed effects, it does show that overexpression of Akt up-regulates the expression of candidate soluble molecules. The direct injection of thymosin β4 has recently been reported to limit myocardial infarct size and to induce functional recovery in the same experimental model used in our study (28). In addition to its growth-promoting and anti-apoptotic actions, IGF-I has been reported to increase cardiomyocyte contractility in vitro (29) and to improve myocardial function both in normal and infarcted adult rat hearts (30, 31). It is unclear whether Akt overexpression also results in the activation of paracrine factors typically not expressed by the MSCs or simply increases the production of pre-existing factors from low basal levels. Our data on the candidate factors clearly support the latter mechanism, but more studies are needed to address this question. We cannot even exclude the possibility that unidentified novel secreted factors are contributing to the beneficial effects described herein. Although beyond the scope of the current work, a multigenomic/proteomic and functional approach with the aim to elucidate all the factors and pathways involved in the therapeutic effects conferred by the conditioned medium is a high priority considering the biological and therapeutic implications of our discovery.

In conclusion, our study suggests that Akt-MSCs exert direct salutary effects on ischemic cardiomyocytes via paracrine mediators. The therapeutic benefits of Akt-MSCs, at least in the acute phase of myocardial infarction, appear to be attributable primarily to diffusible factors released by the cells that, acting in a paracrine fashion, limit infarct size, and improve ventricular function. The future identification of the exact nature and mechanism of action of the secreted factors may have important implications on the development of novel molecular therapies for the prevention of ischemic tissue damage. Fj

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