Supportive Interaction Between Cell Survival Signaling and Angiocompetent Factors Enhances Donor Cell Survival and Promotes Angiomyogenesis for Cardiac Repair

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Abstract—Akt is a major cell survival and angiogenic mediator downstream of angiopoietin-1 (Ang-1)/Tie-2 signaling pathway. We hypothesize that transplantation of mesenchymal stem cells (MSCs) co-overexpressing Ang-1 and Akt lead to better prognosis. Ang-1 and Akt genes were adenovirally transduced into MSCs from male Fischer rats. Cytoprotective effects of transgene overexpression in vitro were assessed by exposure of cells to 8 hours of anoxia. TUNEL and measurement of lactate dehydrogenase showed that MSCs co-overexpressing Ang-1 and Akt (MAAs) were more resistant to anoxia as compared with the nontransduced MSCs or those transduced with Ang-1 or Akt alone. For in vivo studies, after permanent coronary artery occlusion, animals were grouped (n=20/group) to receive intramyocardial injections of 70 μL of basal medium without cells (group 1) or containing 3×10⁶ nontransduced MSCs (group 2) or MAAs (group 3). Four animals per group were euthanized on 4, 7, and 14 days after cell transplantation for molecular studies. Extensive survival of MAAs was observed in group 3, which continued to co-overexpress transgenes in rat heart at 2 weeks after cell transplantation. Immunohistology at 4 weeks revealed myogenic differentiation of donor cells at the site of cell graft. Blood vessel density was highest in the infarct and perinfarct regions in group 3 (P<0.05). Echocardiography at 4 weeks showed that heart function indices were significantly improved in group 3 (P<0.05), including ejection fraction and fractional shortening as compared with groups 1 and 2. We conclude that supportive interaction between Ang-1 and Akt during MSC transplantation gave better prognosis via enhanced cell survival, improved angiomyogenesis, and restored global cardiac function. (Circ Res. 2006;99:776-784.)

Key Words: adult stem cells ■ Akt ■ angiogenesis ■ angiopoietin-1 ■ heart

Bone marrow (BM)-derived mesenchymal stem cells (MSCs) possess multilineage transdifferentiation potential.1,2 When grown under a specific set of cell culture conditions or after transplantation in animal model, they differentiate to adopt cardiac phenotype.3,4 With an emerging interest to combine cell transplantation with gene therapy, MSCs are being assessed for their potential as carriers of exogenous therapeutic genes. Transplantation of MSCs transduced with vascular endothelial growth factor (VEGF) gene in a rat model of coronary artery occlusion showed significantly enhanced collateral formation and improved regional blood flow in the ischemic myocardium.5 Similarly, MSCs overexpressing Akt reduced collagen deposition and attenuated infarct size in rat heart model by inhibition of cardiomyocyte apoptosis in the perinfarct area.6 There was significant inhibition of the remodeling process and complete normalization of systolic and diastolic functions. This improvement was attributed to the activation of various protein factors downstream of Akt cell survival signaling pathway. Similar results were duplicated in a pig heart model of myocardial infarction (MI) after transplantation of MSCs expressing Akt.7 In both of these studies, manipulation of MSCs for Akt gene overexpression resulted in their enhanced survival and better engraftment. We argue that the better engraftment of donor cells may be less meaningful if regional blood flow in the ischemic myocardium is not restored. For an ideal therapeutic intervention, survival and engraftment of the donor cells and reperfusion of ischemic myocardium by angiogenesis are the optimal requirements. These end points may only be achieved by combining stem cell transplantation with appropriate therapeutic transgene delivery to the ischemic myocardium. The rationale behind our present study was to ensure maximum cell survival and enhanced angiogenesis by the delivery of Ang-1 and Akt genes for cardiac repair. Indeed, there is an emerging role for Ang-1, an agonist of Tie-2 receptor, in angiogenesis and a central role for Akt, a member of the class of serine or threonine protein kinases downstream of Ang-1, in cell survival signaling. Hence, they together form an optimal combination of transgenes, the simultaneous expression of which will expectedly lead to

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successful engraftment of donor cells and increased angiogenesis. We hypothesize that transplantation of MSCs co-overexpressing Ang-1 and Akt (MAAs) prevents apoptosis and enhances angiogenesis, together with their myogenic differentiation in the infarcted myocardium.

Materials and Methods

Purification and Expansion of MSCs

BM was obtained from young male Fischer-344 rats by flushing the cavity of femurs and tibia with basal DMEM cell culture medium. BM cells were seeded into 150-mm dishes and cultured in DMEM supplemented with 15% FBS and antibiotics. A small number of cells developed into visible symmetric colonies by 6 to 8 days. Nonadherent hematopoietic cells were removed during routine fresh medium replacement. The adherent, spindle-shaped MSCs were expanded and cultured for no more than 2 to 3 passages before transplantation.

Flow Cytometry

Ex vivo–expanded MSCs were analyzed by flow cytometry (FACSCalibur, BD) for their surface marker expression. The cells were detached with cell dissociation solution (SIGMA) and washed with buffer containing 1% bovine serum albumin (Sigma). After blocking for nonspecific binding with buffer containing 10% FBS, the cells were incubated for 30 minutes at 4°C with the FITC-conjugated antibodies against rat CD34 (Santa Cruz), CD45, CD29 (BD Pharmingen), CD90 (AbCam), and CD117 (Chemicon). The labeled cells were analyzed by flow cytometry using isotype-identical antibodies as controls. At least 1×10^6 cells per sample were acquired and analyzed.

Viral Vector Propagation and Transduction of MSCs

The adenoviral vector (Ad) without therapeutic gene (Ad-null) and the one encoding for Ang-1 (Ad-Ang-1) were provided by Dr Ge Ruowen (National University of Singapore). Ad-vector for myristylated Akt (Ad-Akt) was provided by Dr Meifeng Xu (University of Cincinnati). Ad-vector encoding for lac-z reporter gene with nuclear localization signal (nlslac-z) was purchased from Gene Transfer Vector Core (University of Iowa). These replication-deficient vectors were propagated in 293 cells using DMEM supplemented with 15% FBS. For measurement of infarction size and area of fibrosis, the heart was exposed via minimal left-sided thoracotomy. Left anterior descending (LAD) coronary artery was occluded using Prolene no. 6-0 suture. Immediately after that, the animals were grouped (n=20/group) to receive injections of 70 μL of basal DMEM without cells (group 1) or containing 3×10^6 nontransduced MSCs (group 2) or 3×10^6 MAAs (group 3), respectively. The intramyocardial injections were performed at multiple sites (4 to 5 sites per animal) in the free wall of the left ventricle. The chest of the animals was sutured and the animals were allowed to recover.

Experimental Model of Acute MI and Cell Transplantation

Young female Fischer-344 rats (n=60) each weighing 180 to 200 g were used in this study. The present study conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985) and protocol approved by the Institutional Animal Care and Use Committee, University of Cincinnati.

Histochemical and Immunohistochemical Studies

For measurement of infarction size and area of fibrosis, the heart was arrested in diastole by intravenous injection of cadmium chloride and fixed using formalin. The heart was excised, cut transversely, and embedded in paraffin. Histological sections of 6-μm thickness were cut and used for hematoxylin/eosin and Masson’s trichrome staining. ELISA for Ang-1

Supernatant samples from MSCs and MAAs were collected at 2-day intervals until 15 days after transduction and analyzed with the Quantikine Human Angiopoietin-1 Immunoassay (R&D Systems) according to the instructions of the supplier.

Cytoprotective Effects of Transgene Overexpression Against Oxygen and Glucose Deprivation

MAAs and MSCs transduced with either Ad-null or Ad-Ang-1 or Ad-Akt were grown at a cell density of 3×10^6 cells/Petri dish for 24 hours. For oxygen and glucose deprivation (OGD) treatment, cell culture medium was replaced with glucose and serum-free DMEM. The cells were placed in a 37°C airtight anaerobic chamber (Forma Scientific Corp) saturated with 95% N2/5% CO2. For normoxic control, the cells were maintained at 37°C/5% CO2 incubator. After 8 hours of incubation, the supernatant from each Petri dish was removed for lactate dehydrogenase (LDH) analysis while the cells were harvested and labeled with annexin-V/propidium iodide (PI) using the Annexin-V FITC Apoptosis detection kit (Sigma) for fluorescence-activated cell sorting (FACS).

Labeling of Donor Cells

For their identification and tracking, cells were either labeled with nlslac-z reporter gene by Ad-vector transduction as described previously or with PKH-26 red fluorescent cell tracker dye using Red Fluorescent Cell Linker Kit (Sigma) per the instructions of the manufacturer.

Physiologic Assessment of Heart Function

Transhoracic echocardiography was performed to study change in the heart function at 4 weeks after respective treatment. Each animal was anesthetized and placed in supine position. The heart was imaged in 2D and M-Mode, and recordings were obtained from parasternal long axis view at papillary muscles level using Compact Linear Array probe CL10–5 on an HDI-5000 SONOS CT. Anterior and posterior end-diastolic and end-systolic wall thickness and left ventricular (LV) internal dimensions and LV end-systolic (LVESD) and end-diastolic (LVEDD) diameters were measured from at least 3 consecutive cardiac cycles. Indices of LV systolic functions, including LV fractional shortening (LVFS) and LV ejection fraction (LVEF), were calculated using LVFS=(LVEDD−LVESD)/LVEDD×100 and LVEF=[(LVEDD^2−LVESD^2)/(LVEDD^2)]×100 relations, respectively, and the results were expressed as percentage.

Histochecmical and Immunohistochemical Studies

For measurement of infarction size and area of fibrosis, the heart was arrested in diastole by intravenous injection of cadmium chloride and fixed using formalin. The heart was then excised, cut transversely, and embedded in paraffin. Histological sections of 6-μm thickness were cut and used for hematoxylin/eosin and Masson’s trichrome staining.
staining for visualization of muscle architecture and thickness of the LV wall as described earlier.\(^9\) Infarct size was defined as the sum of the epicardial and endocardial infarct circumference divided by the sum of the total LV epicardial and endocardial circumferences using computer-based planimetry with ImageJ analysis software (version 1.6065; NIH). For histochemical analysis of β-galactosidase activity, X-gal staining was performed on nlslac–z–transduced cells or tissue sections as described earlier.\(^8\) The nlslac–z–positive tissue sections were counter-immunostained for transgene expression after transplantation using our routine protocols.

Blood vessel density was assessed as previously described.\(^8\) Briefly, cryosections (6-μm thickness) were immunostained using specific antibodies for von Willebrand factor VIII (vWF-VIII) and detected with fluorescein isothiocyanate (FITC)-labeled secondary antibody. In some tissue sections, counter-immunostaining was performed for smooth muscle actin (SMA) and detected by specific secondary antibody conjugated with Alexa Fluor-350 (Molecular Probes). The number of blood vessels positive for vWF-VIII and SMA were counted in both infarct and periinfarct regions. At least 2 microscopic fields each in infarct and periinfarct regions were randomly selected and counted in at least 2 sections from each animal (n=4 animals per group). Blood vessel density was expressed as the number of vessels per microscopic field (x200). Blood vessel maturation was assessed by calculating SMA-positive blood vessels in relation to the vWF-VIII–positive vessels.

**Terminal dUTP Nick-End Labeling Assay**

TUNEL was performed on cells or deparaffinized 5-μm-thick sections with an In-Situ Cell Death Detection kit (Roche Inc) per the instructions of the manufacturer. 4′,6-diamidino-2-phenylindole (DAPI) staining was performed to stain the nuclei. The degree of apoptotic cell death was determined by counting the total number of TUNEL-positive nuclei per microscopic field (x400).

**Statistical Analysis**

All data were described as mean±SEM. To analyze the data statistically, we performed Student’s t test and 1-way ANOVA with post hoc analysis. A value of P<0.05 was considered statistically significant.

**Results**

**MSC Purification, Propagation, and In Vitro Studies**

The plastic adherent MSCs were propagated for 2 to 3 passages before their characterization for the surface marker expression and transplantation into rat model of acute MI. The isolated cells were 95.16% pure for CD29, 86.3% for CD44, 79% for CD90, and 0.95% for CD117 (supplemental Figure IA). The contaminating populations of hematopoietic stem cells positive for CD34 and CD45 were 1.6% and 3.8%, respectively.

Transduction of MSCs with Ad-vector constructs encoding for Ang-1 and Akt was performed successfully. More than 90% of these cells concurrently overexpressed Ang-1 and Akt (supplemental Figure I, B through D). RT-PCR showed that the transgene expression in vitro continued until 15 days of observation (supplemental Figure IE). Ang-1–specific ELISA was performed on the cell culture supernatant samples collected from MAAs, using cell culture supernatant from nontransduced MSCs as control. Peak level secretion of Ang-1 (15.19 ng/mL) was observed between 4 to 6 days from the start of the transduction procedure (supplemental Figure IF).

**Cytoprotective Effects of Transgenes Expression**

LDH release from cells after 8 hours in OGD was used as an indicator of cellular injury. MAAs showed highest level of resistance to OGD, whereas Ad-null–transduced MSCs...
showed highest sensitivity. LDH levels were highest in the Ad-null–transduced MSCs as compared with MAAs and MSCs overexpressing either Ang-1 or Akt alone (Figure 1A). Similarly, annexin-V/PI staining analyzed by FACS showed the highest annexin-V/PI-negative cells in MAAs (Figure 1B through 1F). Taking these results into consideration, MAAs were used for heart cell therapy in experimental rat model of acute myocardial infarction.

Animal Surgery
All of the animals survived the full length of the experiment. There were no deaths related with cell transplantation. Twelve animals died during the creation of MI. Four animals per group were euthanized on 4, 7, and 14 days after cell transplantation for molecular and histological studies.

Survival of Donor Cells In Vivo and Myogenesis
Transduction efficiency for nlslac-z reporter gene into MSCs was more than 95% (supplemental Figure IIA), and labeling efficiency with PKH-26 cell tracker dye was more than 99%. These cells were injected immediately after LAD coronary artery ligation. Four days after cell transplantation, histochemical analysis for β-galactosidase activity in the cell transplanted heart tissue sections revealed extensive survival of nlslac-z–positive cells at the site of the cell graft. Counter-immunostaining of the nlslac-z–positive tissue sections for human Ang-1 and Akt expression showed that the surviving MAAs continued to express these transgenes (supplemental Figure IIB). These results were further confirmed by RT-PCR conducted on the rat heart tissues taken from various treatment groups (supplemental Figure IIC). A significantly higher number of the TUNEL-positive nuclei were observed in group 2 animal hearts as compared with group 3 (Figure 2A through 2G). These observations were in conformity with our in vitro data, which showed better survival of MAAs when subjected to OGD for 8 hours (Figure 1A through 1F).

Histochemical staining for nlslac-z expression in rat heart tissues euthanized at 2 weeks after cell transplantation showed that the nlslac-z–positive cells were incorporated predominantly into the center and border zone of infarcts, whereas fewer of these cells were detected in the noninfarcted myocardium (Figure 3A through 3E). There was an obvious trend for the transplanted cells to show preferential engraftment in the necrotic tissue and adherence with the collagen matrix. Confocal imaging after immunofluorescent staining of rat heart tissues sections for actinin showed that the donor cells engrafted well in the host infarct and periinfarct myocardium (Figure 4A through 4D). Many of the engrafted cells became enlarged and stained positive for the muscle-specific proteins and showed sarcomeric organization as can be seen at higher magnification (Figure 4E and 4F). These data suggest the ability of MAAs to engraft in the ischemic myocardium and undergo myogenic differentiation.

Evidence of Angiogenesis in the Infarcted Myocardium
Angiogenic effect of Ang-1 and Akt co-overexpression was determined by fluorescent immunostaining specific for vWF-VIII expression to detect endothelial cells. Capillary density observed in the center and border area of the infarct in group 3 (76.76±8.00 and 180.75±12.77; P<0.05) and group 2 (63.87±2.73 and 95.76±3.6;
was significantly higher than that of group 1 (41.05 ± 1.3; 66.4 ± 3.55) as shown in Figure 5A through 5G. We did not find angioma formation in the transplanted hearts with nontransduced MSCs or MAAs, either grossly or by microscopic examination. Double fluorescent immunostaining for vWF-VIII and SMA revealed that most of the newly formed vessels were mature, having a SMA covering (supplemental Figure III, A through F).

Figure 3. A through E, Histochemical staining of rat heart tissue sections for nlslac-z expression at 2 weeks after cell transplantation. Extensive survival of the donor cells was observed in both infarct and periinfarct regions in group 3 (A and B) as compared with group 2 (D and E). Group 1 served as a control and did not show nlslac-z expression (1C). Magnification: ×100 (A and D); ×300 (B, C, and E).

Histochemical staining showed that the transplanted nlslac-z–expressing donor cells integrated into the blood vessels, both in the infarct as well as periinfarct regions (Figure 6A through 6D). Similar results were obtained from the confocal images after vWF-VIII immunostaining. These results revealed that some of the transplanted PKH-26–labeled cells were found positive for vWF-VIII expression.

Figure 4. A through F, Confocal images of rat hearts tissue sections from group 3. The animals were euthanized at 4 weeks after PKH-26–labeled MAA transplantation. The 5-μm-thick frozen tissue sections were immunostained using actinin-specific primary antibody and detected by FITC (green fluorescence) labeled secondary antibody. DAPI (blue fluorescence) was used for visualization of the nuclei. Figure 4E and 4F show magnified regions of the infarct and periinfarct areas showing development of the new muscle fibers from the transplanted PKH-26–labeled cells (white arrows). Magnification, ×630.
and participated in the formation of vascular structures in the myocardium (Figure 7A through 7E).

**Infarct Size and Area of Fibrosis**

The hearts of all animals had large anterolateral wall infarctions. Cross-sections at mid-papillary muscle level showed transmural infarctions in all the animals. Marked left ventricle wall thinning was observed in group 1 (supplemental Figure IV, A through D). On the other hand, groups 2 and 3 showed comparatively better maintenance of the wall thickness. The size of the infarct was significantly reduced in group 3 ($\text{P}<0.05$) as compared with group 1 ($44.61 \pm 2.6$). Severe fibrosis of the myocardium was observed in group 1, but significantly smaller fibrosis was observed in group 2 and group 3 at 4 weeks after respective treatment. The area of fibrosis in group 3 animals was $25 \pm 2.4$ ($\text{P}<0.05$) as compared with the group 2 ($27.9 \pm 1.2$; $\text{P}<0.05$) and group 1 ($35 \pm 2.6$).

**Assessment of the Heart Function**

Four weeks after cell implantation, the systolic function indices including LVEF and LVFS showed significant improvement in group 2 (55.85 $\pm 2.51$, $\text{P}<0.05$; 24.04 $\pm 1.42$, $\text{P}<0.05$) and group 3 (68.4 $\pm 2.53$, $\text{P}<0.05$; 32.3 $\pm 1.77$, $\text{P}<0.05$) animals as compared with group 1 (Figure 8A through 8B). There was significant reduction in LVEF and LVFS in group 1 during the same time of observation. LVEF and LVFS between group 3 and group 2 also showed significant

![Figure 5](https://example.com/figure5.png)

**Figure 5.** A through G, Effect of transgene expression on capillary density in rat heart at 4 weeks after cell transplantation. (FITC green fluorescence for vWF-VIII expression.) Significantly increased number of blood vessels was observed in both periinfarct and infarct areas in group 3 ($\text{P}<0.05$) and group 2 ($\text{P}<0.05$) as compared with group 1. Data represent mean $\pm$ SEM. Magnification $\times 200$.

![Figure 6](https://example.com/figure6.png)

**Figure 6.** A through D, Histochemical staining of rat heart tissue for nls-lac-z expression at 2 weeks after MAA transplantation. The cryosections (6-μm thickness) were stained by X-gal staining method (as described in Materials and Methods). The transplanted cells can be seen integrated into the blood vessel of periinfarct (A and B) and infarct (C and D) area. Magnification A and C = $\times 400$; B and D = $\times 100$. 
difference ($P<0.05$). LVESD was significantly smaller in both group 3 (0.46±0.023; $P<0.05$) and group 2 (0.51±0.021; $P<0.05$) as compared with group 1 (0.62±0.012) (Figure 3C and 3D). Although we did not observe any appreciable change in LVEDD of the 3 animal groups, there was a trend showing reduced LVEDD in group 3 and group 2 as compared with group 1. These data clearly indicated that MAAs exerted greater cardioprotective effects as compared with nontransduced MSCs. However, transplantation of MSCs, either transduced or nontransduced, attenuated LV remodeling and preserved the cardiac function better than the DMEM injection without cells.

**Discussion**

In this study, we have shown that co-overexpression of Ang-1 and Akt produced powerful cytoprotection against OGD in vitro. Moreover, transduction with Ang-1 and Akt genes resulted in marked survival of the transplanted MSCs in vivo, their differentiation into myocytes and participation in neo-vascularization. This resulted in the reduced infarct size and optimally preserved cardiac function after MI.

Despite encouraging results emanating from the preclinical as well as clinical studies, donor cell survival after engraftment remains among the major limitations that significantly influence the outcome of heart cell therapy. In some of the published reports, donor cell survival between 0 to 1 hour after injection averaged up to only 58%. Similarly, as low as 1% of the donor cells survived during first 24 hours after transplantation. Although the underlying molecular mechanism and kinetics of donor cell death in the infarcted myocardium are undefined, apoptosis is a major factor in their demise. Some studies have shown the immune-privileged nature of BM cells after transplantation into immunocompetent and immunosuppressed hosts. These observations thus leave host immune response–unrelated factors as the major players responsible for the massive cell death and underscore the need for strategies to limit these factors. This may include protection against mechanical stress, bouts of ischemia, ischemia/reperfusion, and more importantly the host inflammatory response mediators and proapoptotic factors in the ischemic myocardium. Heat shock imparts cytoprotection via stabilization of various aspects of cell metabolism and function. In a recently published study, transduction of MSCs with hypoxia-regulated heme oxygenase-1 improved cell graft survival in the ischemic myocardium. With Akt having a central role in cell survival signaling, its activation exerted a powerful cardioprotection after transient ischemia, inhibited cardiomyocyte death and improved function of the surviving cardiomyocytes. Transplantation of MSCs expressing Akt in a rat heart model restored 4-fold greater myocardial volume as compared with nontransduced MSCs. Recently, we have observed that preconditioning of MSCs enhanced their survival and their ability to attenuate LV remodeling, which was in part mediated by paracrine effects. This study is a modification of our strategy to achieve cytoprotective effects during acute...
phase of MI by transplantation of MAAs. Besides, we anticipated that activation of Ang-1 and Akt would orchestrate the signaling pathways potentially involved in angiogenesis and cell survival to salvage the host myocardium at risk, in combination with the functional benefits of MSC-based cellular cardiomyoplasty. Our rationale in the combined therapeutic approach was that the beneficial effects of enhanced donor cell survival might not be translated in reality unless regional blood flow in the ischemic myocardium was restored. Angiogenesis optimally preserves myocardial function by maintenance of the viability of the residual cardiomyocytes and grafted cells.\(^17\)

Using the cell-based multiple gene therapy approach, we have already shown that skeletal myoblasts co-overexpressing Ang-1 and VEGF gave enhanced blood vessel density in rabbit and rat models of hindlimb ischemia and MI, respectively.\(^18,19\) In an attempt to formulate an ideal gene combination for therapy, synergistic interaction between insulin-like growth factor and VEGF transgenes gave improved cytoprotection.\(^20\) Our choice of Ang-1 and Akt transgene combination will allow us to achieve maximum beneficial effects on cell survival and angiogenesis. Ang-1 has a pivotal role as the modulator of vascular development.\(^21\) In vitro studies have shown that Ang-1 can cause sprouting of new capillaries in vitro\(^22\) and can promote stable and leak resistant blood vessels formation in vivo.\(^21,23\) More importantly, Ang-1 enhances endothelial cell survival via the phosphatidylinositol 3-kinase (PI3K) pathway.\(^24,25\) Recent studies have depicted a major role for Akt downstream of Ang-1/Tie2 signaling pathway and is the primary mediator of endothelial cell survival via Akt/FKHR transcription factor.\(^26,27\) Constitutive expression of Akt enhanced cardiomyocyte survival in culture from reperfusion injury.\(^28\) Akt exerts dual effect on the myocardium by increasing the cell size and improving contractility.\(^29\) These activities of Akt have been exploited for better survival of donor cells and for the ischemic cardiomyocytes in heart cell therapy. In our present study, Ang-1 and Akt co-overexpression rendered MAAs more resistant to ischemia and improved their survival when subjected to anoxia in vitro as well as after transplantation.

The time of donor cell delivery after MI episode has a decisive role in their survival.\(^30\) The cytokine rich myocardial microenvironment together with the infiltrating cells as part of the inflammatory cascade in response to ischemic injury may be hostile to the transplanted cells. There is likelihood that the cells will not thrive under these unfriendly conditions without preconditioning to become more resilient. We decided to administer cells to the animals earlier than many of the other reported studies\(^5,20\) (ranging from 1 hour to several days after MI) for 2 reasons. Firstly, we intended to ascertain the protective effects of the overexpressed transgenes during the time period after MI when peak level intrinsic inflammatory response was expected. Secondly, early engraftment of cells may help to salvage more of the myocardium at risk as compared with the delayed engraftment. We observed significantly less TUNEL positivity at the site of the cell graft in group 3 as compared with groups 1 and 2, which was in line with our in vitro observations. In addition, we observed higher number of nlslac-z–expressing cells in the group 3 animal hearts after 2 weeks. Confocal images after immunostaining for muscle-specific structural proteins clearly demonstrated that the engrafted cells underwent myogenic differentiation and formed mature muscle fibers in the recipient myocardium. The enhanced survival of donor cells undergoing myogenic differentiation led to reduced infarct size and increased LV wall thickness. Further studies are necessary, however, to elucidate the contractile nature of the newly formed muscle fibers.

The therapeutic potential of MSCs has also been attributed to their ability to adopt vascular endothelial phenotype and participate in angiogenesis. We performed a comprehensive evaluation of the distribution profile of angiogenesis in the recipient heart. Blood vessel density dominated at the site of cell graft and highest density of newly formed vessels was observed in the perifarct region in group 3. There was an obvious propensity of vWF-VIII–positive cells in group 3 heart tissue sections as compared with the other 2 animal groups. Besides, our results clearly showed that there was a spatial distribution of nlslac-z–expressing donor cells, getting integrated into the blood vessels both in the infarct as well as perifarct areas. This may be explained by the multilineage potential of MSCs, which allowed their incorporation into blood vessels by undergoing in situ differentiation.\(^31\) These results are in harmony with the results from other research groups that showed that MSCs lack in endothelial cell surface marker expression in undifferentiated state. However, they can adopt endothelial cell phenotype, migrate to the vessel wall and luminal endothelium, and participate in microvascular network remodeling.\(^32\) PKH-26–labeled MAAs immunostained positively for vWF-VIII and were integrated into the blood vessel architecture. Taken together, the improved cell survival in vivo together with angiomyogenesis achieved from the transplanted cells gave better heart function and remodeling.

**Study Limitations**

Despite convincing evidence that our transplanted MSCs participated in neomyogenesis and angiogenesis, which led to the improved cardiac function, the study has some limitations. Cutting back on these limitations will refine and further enhance the effectiveness of our therapeutic approach. The in vitro data showed that virally transduced MSCs were more sensitive to ischemia as compared with the nontransduced cells. Use of the nonviral vector for transgene delivery may alleviate this concern. Moreover, there is a need to assess the stability of these beneficial effects over longer time period.

In summary, MAAs showed extensive survival and engraftment in the infarcted rodent heart. The activation of multiple downstream substrates of Ang-1 and Akt converge to prevent the induction of apoptosis in the early phase and interfered with biological functions of the endothelial cells, which contribute to vascular remodeling and vessel integrity during angiogenesis. Furthermore, the engrafted MAAs underwent myogenic differentiation, participated in angiogenesis, and resulted in improved LV contractile function.

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Disclosures

None.

References


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In an article by Jiang et al. (Circ Res. 2006;99:776-784), “Supportive Interaction Between Cell Survival Signaling and Angiocompetent Factors Enhances Donor Cell Survival and Promotes Angiomyogenesis for Cardiac Repair,” figure 3 was incorrectly reproduced at a low resolution, rendering parts of the figure unreadable. The figure appears below in high-resolution format, as the authors intended. The corrected article is now available at http://circres.ahajournals.org. The Publisher regrets this error.

**Figure 3.** A through E, Histochemical staining of rat heart tissue sections for nlslac-z expression at 2 weeks after cell transplantation. Extensive survival of the donor cells was observed in both infarct and perinfarct regions in group 3 (A and B) as compared with group 2 (D and E). Group 1 served as a control and did not show nlslac-z expression (1C). Magnification: ×100 (A and D); ×300 (B, C, and E).
Detailed Methods

**Double fluorescent immunostaining of cells**

MSC and MAA were grown in glass chambers and fixed with -20°C cold methanol. The cells were incubated at 37°C with mouse anti-Ang-1 (Sigma, USA) and rabbit anti-Akt (Cell Signaling Inc., USA) primary antibodies. After washing, they were incubated for 1 h with respective secondary antibody conjugated with Alexa Fluor-555 and Alexa Fluor-488 (Molecular Probes) respectively. Various microscopic fields were counted for stained and unstained cells using a fluorescent microscope (BX41 Olympus, Japan) fitted with a digital camera (Olympus).

**RT-PCR of MAA for Ang-1 and Akt expression in vitro and in vivo**

RNeasy kit (QIAGEN) was used for isolation of total RNA from the cells or rat heart tissue samples as per supplier’s instructions. *(Detailed of the method is available as supplement data)* For reverse transcription, first strand cDNA was synthesized from the isolated total RNA using Omniscript RT-kit (QIAGEN) as per manufacturer’s protocol. For PCR, 1µg of cDNA from the finished reverse-transcription reaction was added to PCR mix containing Taq DNA polymerase. Template DNA was amplified using the following thermocycle profile: initial denaturation for 3 min at 94°C; the 3-step cycling of denaturation for 1 min at 94°C, annealing at 63.7 for 1 min and extension for 1 min at 72°C, repeated for a total of 30 cycles; and a final extension for 10 min at 72°C.

Legends to the Supplemental Figures

**Supplemental Figure 1A-F:** In vitro characterization. (A) FACS histogram of MSC for CD117 expression (B-D) Double fluorescent immunostaining of MAA for co-
overexpression Ang-1 and Akt in vitro (red fluorescence= Akt; green fluorescence= Ang-1). (E) ELISA for Ang-1 secretion from MAA in vitro and (F) RT-PCR of MAA for Ang-1 and Akt overexpression in vitro until 15 days of observation.

Supplemental Figure 2A-C: (A) Histochemical staining of MSC for nls lac-z expression localized in the nucleus. (B) Immunostaining of lac-c positive rat heart tissue sections for expression of Akt at the site of the cell graft 4 days after MAA transplantation (green colour = x-gal staining for lac-z expression; dark brown colour= Primary reaction of antibody specific for Akt visualized by specific secondary antibody conjugated with horse raddish peroxidase enzyme reaction with diaminobenzidine substrate (C) RT-PCR analysis of rat myocardium for simultaneous expression of Ang-1 and Akt in vivo 4 days after MAA transplantation.

Supplemental Figure 3A-F: Double fluorescent immunostaining of the infarcted rat heart in group-3 at 4 weeks after cell transplantation. Frozen tissue sections of 6µm thickness were immunostained using vWFactor-VIII and smooth muscle actin (SMA) specific primary antibodies and were visualized with specific secondary antibodies conjugated with FITC (green fluorescence) and Alexa Flor-455 (blue fluorescence). Figures A-C represent the infarct area whereas D-F represent peri-infarct area.

Supplemental Figure 4A-D: Histological studies of the heart at 4 weeks after coronary artery ligation and cell transplantation in different animal groups. Paraffin tissue sections of the heart were stained by Mason trichome staining. Infarct size and area of fibrosis...
(p<0.05) were significantly reduced in MAA group-3 as compared with DMEM group-1.

Values are mean± SEM.
Supplemental Figure 1A-F:

*Each point represents an average of three readings.*
Supplemental Figure 2A-C
Supplemental Figure 3A-F

Infarct area

Peri-Infarct area
Supplemental Figure 4A-D