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IFATS Collection: Human Adipose Tissue-Derived Stem Cells Induce Angiogenesis and Nerve Sprouting Following Myocardial Infarction, in Conjunction with Potent Preservation of Cardiac Function

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Abstract

The administration of therapeutic cell types, such as stem and progenitor cells, has gained much interest for the limitation or repair of tissue damage caused by a variety of insults. However, it is still uncertain whether the morphological and functional benefits are mediated predominantly via cell differentiation or paracrine mechanisms. Here, we assessed the extent and mechanisms of adipose-derived stromal/stem cells (ASC)-dependent tissue repair in the context of acute myocardial infarction. Human ASCs in saline or saline alone was injected into the peri-infarct region in athymic rats following left anterior descending (LAD) coronary artery ligation. Cardiac function and structure were evaluated by serial echocardiography and histology. ASC-treated rats consistently exhibited better cardiac function, by all measures, than control rats 1 month following LAD occlusion. Left ventricular (LV) ejection fraction and fractional shortening were improved in the ASC group, whereas LV remodeling and dilation were limited in the ASC group compared with the saline control group. Anterior wall thinning was also attenuated by ASC treatment, and post-mortem histological analysis demonstrated reduced fibrosis in ASC-treated hearts, as well as increased peri-infarct density of both arterioles and nerve sprouts. Human ASCs were persistent at 1 month in the peri-infarct region, but they were not observed to exhibit significant cardiomyocyte differentiation. Human ASCs preserve heart function and augment local angiogenesis and cardiac nerve sprouting following myocardial infarction predominantly by the provision of beneficial trophic factors.

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Disclosure of Potential Conflicts of Interest

B.H.J. and K.L.M. have performed contract work for Lilly.

Keywords

Adipose tissue-derived stem cell; Myocardial infarction; Heart function; Angiogenesis

Introduction

Early clinical trials have demonstrated that autologous bone marrow and peripheral blood-derived stem and progenitor cells are safe and potentially efficacious treatments for ischemic heart disease [1–3]. The majority of trials have evaluated autologous cells to avoid issues of immunotolerance. However, therapeutic cells extracted from bone marrow or peripheral blood after mobilization are available in limited numbers, cannot be easily expanded, and, at least for bone marrow, are obtained using invasive and often painful methods. Therefore, a significant hurdle that must be overcome to develop a widely adopted and robust therapy is the identification of an easily accessible source of therapeutic cells that are available in large numbers.

The discovery that the nonadipocyte, stromal fraction of adipose tissues contains an abundant population of multipotent stem cells that can be easily harvested in high numbers by minimally invasive surgical techniques has suggested a novel source of cells for therapeutic use. Adipose-derived stromal/ stem cells (ASCs) include cells possessing the ability to differentiate into multiple mesenchymal cell types in vitro, including endothelial cells [4–6] and cardiomyocytes [7–9]. In addition to their potential capacity for tissue regeneration via plasticity, we have shown that ASCs also secrete bioactive levels of many potent growth factors and cytokines, which are critically important for repair of ischemic injury [10–13].

In this study we evaluated the ability of unmodified human ASCs to promote restoration of heart function in a chronic myocardial infarction model in athymic nude rats. We demonstrate the therapeutic potential of ASCs for myocardial diseases by a remarkable effect of limiting structural and functional myocardial damage. We present evidence that the cells persist for up to 1 month in the myocardium, predominantly in infarct border zones, but do not display evidence of transdifferentiation and direct incorporation into regenerating myocardial tissue. Rather, the long-term engraftment of ASCs is associated with increased angiogenesis, as well as the novel finding of increased nerve sprouting, in regions of cell delivery. These findings, taken together, suggest that ASCs limit injury and promote function of cardiac tissues predominantly by provision of trophic support to stimulate cell survival and regeneration, including myocardial, vascular, and neural structures.

Materials and Methods

Isolation and Culture of Human ASCs

Human subcutaneous adipose tissue samples were obtained following routine, elective lipoaspiration of a 50-year old healthy female patient with a body mass index of 26 under approval from the Indiana University Institutional Review Board. The lipoaspirate was digested in collagenase type I solution (Worthington Biochemical, Lakewood, NJ, http://www.worthington-biochem.com) under gentle agitation for 1 hour at 37°C, filtered with 500- and 250-μm Nitex filters (Sefar Printing Solutions, Inc., Lumberton, NJ, http://www.sefar-screens.com), and centrifuged at 200*g* for 5 minutes to separate the stromal cell fraction (pellet) from adipocytes. The ASC fraction was centrifuged at 300*g* for 5 minutes. The supernatant was discarded, and the cell pellet was resuspended in endothelial growth medium-2 microvascular (MV) (EGM-2MV; Cambrex, Walkersville, MD, http://www.cambrex.com), which consists of endothelial basal medium-2, 5% fetal bovine serum (FBS), and the supplemental growth factors vascular endothelial growth factor (VEGF),

basic fibroblast growth factor, epidermal growth factor, and insulin-like growth factor-1. Isolated ASCs were cultured for 48 hours, removed from the culture plate by trypsin/EDTA treatment, and resuspended in freezing medium composed of Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, http://www.invitrogen.com), 20% FBS (HyClone, Logan, UT, http://www.hyclone.com), and 10% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com), before being frozen in liquid nitrogen.

Approximately 1.5 weeks before being used in the experiments described below, ASCs were thawed and plated in EGM-2MV. After 2 days in culture, nonadherent cells were removed by washing with phosphate-buffered saline (PBS), and new EGM-2MV was added to the flasks. The ASCs were cultured for two more passages to expand. Cells at passage 3 were detached from the flask by brief treatment with trypsin/EDTA, washed twice with PBS, and resuspended in saline. The number of viable cells was determined by treating with trypan blue dye and counting viable (nonblue) cells using a hemocytometer. A portion of the cells was analyzed by flow cytometry as described in supporting information Fig. 1.

Athymic Nude Rat Model of Myocardial Infarction

The animal studies were approved by the Indiana University School of Medicine Animal Use and Care Committee. Twenty athymic nude rats, ages 7–8 weeks, were anesthetized by isoflurane gas (Abbott, Abbott Park, IL, http://www.abbott.com), a left thoracotomy was performed at the fifth intercostal space, and the proximal left anterior descending (LAD) artery was encircled with a 6-0 Prolene (Ethicon, Inc., Somerville, NJ, http://www.ethicon.com) and permanently ligated. Rats were randomly separated into two groups to receive direct intramyocardial injections of either 10^6 ASCs (>85% viable) or saline alone in 100 μl at two sites in developing peri-infarction zones (identified as the blanching region in the anterioapical region) at 1 hour after ligation. The chest was closed with three layers of sutures, and the rats were placed in cages on top of a warming pad and supplied with oxygen until they had recovered. Buprenorphine (0.1 mg/kg, subcutaneous) was given immediately after surgery and on each of the 3 days following.

Assessment of Heart Function In Vivo

Heart function was assessed at 3 days before and then again at 4 and 28 days after surgery by echocardiography (Vevo770; Visualsonics, Toronto, ON, Canada, http://www.visualsonics.com) [14,15]. The rats were anesthetized and put in a supine position, and gel (Aquasonic 100; Parker Laboratories, Inc., Fairfield, NJ, http://www.parkerlabs.com) was applied liberally to the chest before the transducer (710B) was placed on the area of the precordial impulse (approximately the fourth to fifth intercostal space) and positioned so that a clear image of the heart was obtained without lung or rib interference. Both two-dimensional and M-mode images were recorded, and measurements were calculated using the installed cardiac analysis software package (Visualsonics, version 2.2.3).

Histological Analysis of Infarction-Induced Fibrosis

At the end of the experiment, rats were euthanized with carbon dioxide, and hearts were harvested and then frozen in Tissue-Tek O.C.T. Compound (Sakura Finetek, Torrance, CA, http://www.sakura.com) for subsequent cryosectioning. Sections 8 μm in thickness were stained for tissue morphology (hematoxylin and eosin) plus fibrosis (Masson's trichrome) [16]. Infarct size was calculated as the ratio of the cross-sectional area of infarct wall to the entire cross-sectional area of the left ventricle.

Histological Analysis of Angiogenesis and Nerve Sprouting

Thin sections (8 μm) were probed with biotinylated antibodies to smooth muscle α-actin (A2547; Sigma-Aldrich). Visualization of binding was accomplished by streptavidin-horseradish peroxidase complex formation and followed by color development with 3,3′-diaminobenzidine (D4168; Sigma-Aldrich). Digital images of sections were obtained at ×200 magnification with a Nikon microscope (model TE2000-S; Nikon, Tokyo, http://www.nikon.com) and were analyzed with ImageJ software (NIH). Five fields from each muscle section were randomly selected for quantification of smooth muscle α-actin (SMA)-positive arterioles. The area of the infarct border zone was determined as the 1.5-mm region of histologically intact myocardium surrounding the evident infarct-related fibrocellular region.

Nerve sprouting was assessed by staining thin sections with antibodies to growth-associated protein-43 (GAP43), a marker for nerve sprouting expressed in the growth cones of sprouting axons. A modified immunohistochemical ABC method was used for immunostaining for GAP43 (MAB347; Chemicon, Temecula, CA, http://www.chemicon.com) [17]. The density of stained nerves was determined from digital micrographs using ImagePro software (Mediacybernetics, Bethesda, MD, http://www.mediacy.com) and expressed as the nerve area divided by the total area examined ($\mu m^2/mm^2$), as described earlier [18]. The investigator performing the analysis was blinded to the identity of imaged sections. The nerve density of the border and distal zones of the sectioned hearts was determined by averaging the three fields with the highest nerve density.

Fluorescence Imaging of Tissue Sections

Slide-mounted and acetone-fixed muscle thin sections (5 μm) were preincubated overnight with anti-human human leukocyte antigen (HLA)-ABC (M0736; 1:50; Dako, Glostrup, Denmark, http://www.dako.com) and coincubated with antibodies to Ki67 (Abcam ab27619, 1:50) or α-sarcomeric actin (A2172; 1:500; Sigma-Aldrich). Slides were washed and then incubated with fluorescein-conjugated chicken anti-mouse IgG (A21200; Invitrogen, Carlsbad, CA, http://www.invitrogen.com) or goat anti-rabbit IgG (T2769; Invitrogen) for 30 minutes. Finally, sections were incubated with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; D8417; Sigma-Aldrich) for 1 minute. Images of sections were obtained at ×400 magnification and were analyzed with ImageJ software.

Statistical Analysis

Data are expressed as mean ± SD or SEM (as noted in the text and figures). Statistical comparisons between groups were performed using a two-tailed Student's *t* test. Comparisons of multiple groups were done with analysis of variance, with corrections for multiple comparisons. $p < .05$ was considered to be significant.

Results

ASCs Promote Functional Recovery of Ischemic Myocardium

Serial echocardiographic analysis indicated that heart function in all rats was severely compromised by day 4 following LAD occlusion (Table 1). Significant functional loss continued over the following 24 days in saline-treated hearts. Conversely, hearts injected with ASCs demonstrated a trend toward an increase in function and improved morphometry during the same time interval (Table 1; Fig. 1). As a consequence, 1-month values for all measures of both function and morphometry were significantly improved in ASC-treated hearts compared with saline controls (Table 1). Left ventricular (LV) ejection fraction at 28 days postinfarction was 56% ± 7% (mean ± SEM) with ASC treatment versus 37% ± 3% for saline ($p < .05$).

Correspondingly, fractional shortening was also significantly improved with ASC treatment (32% ± 5% for ASCs vs. 19% ± 2% for saline; $p < .01$). Both end-diastolic and end-systolic LV volumes were lower in the ASC group (311 ± 17 and 139 ± 21 μl, respectively) than in the saline group (391 ± 30 and 249 ± 27 μl) ($p < .05$ and $p < .01$, respectively). Anterior wall thinning measured at end diastole was also attenuated in the ASC group (1.6 ± 0.1 vs. 1.2 ± 0.2 mm; $p < .05$), whereas no difference was observed in posterior wall thickness.

Treatment with ASCs Reduces Infarct Size

To characterize the structural correlates of the favorable effects of ASC injection upon cardiac performance, we evaluated the fraction of LV myocardium that demonstrated infarction in the ASC-treated and control hearts 28 days after inducing permanent ischemia. The fractional region of infarction in ASC-treated hearts (26% ± 6%) was significantly less ($p < .05$) than that in the hearts from the saline group (34% ± 6%) (Fig. 2).

Microvascular Densities Are Significantly Enhanced in Border Zones of ASC-Treated Hearts

Rapid restoration of blood supply to the ischemic region is critical for stabilizing the border region of the infarct and supporting viable and regenerating myocardium. It has been demonstrated previously that ASCs promote revascularization of ischemic tissues via promotion of angiogenesis, both through incorporation into nascent vessels [4,6] and by secretion of paracrine factors to augment vessel formation [10,12,19]. The density of SMA^+ arterioles in thin sections of hearts from ASC- and saline-treated groups was determined (Fig. 3). At 28 days following initiation of the ischemic insult, the density of arterioles in border zone of heart sections in the group treated with ASCs was significantly higher ($p < .05$) compared with saline group (60 ± 9 and 37 ± 2 SMA^+ cells per mm^2, respectively). These data indicate that ASCs injected intramyocardially are able to promote regional vascularization into the border zone of a developing infarct.

ASC Transplantation Induces Cardiac Nerve Sprouting

Myocardial infarction causes cardiac nerve injury and denervation [20]. This may be followed by nerve sprouting in both humans and animals [21–23], which may result in increased cardiac contractile performance. One month after infarction, $GAP43^+$ nerve densities in the infarct border zone region of the LV were actually lower than those in the unaffected posterior wall (Fig. 4). However, by comparison, ASCs induced more nerve sprouting (464 ± 43 $\mu m^2/mm^2$) in the border zone compared with the control group (342 ± 20 $\mu m^2/mm^2$; $p < .05$). There was no significant difference between the two groups in the posterior wall (ASCs, 554 ± 27 $\mu m^2/mm^2$; saline, 582 ± 30 $\mu m^2/mm^2$). To evaluate the factors responsible for such effects, we performed an analysis of proteins secreted by ASCs during culture using antibody arrays. The profile of proteins in medium conditioned by the ASC preparations confirmed the presence of multiple known angiogenic and neurotrophic factors, including VEGF, hepatocyte growth factor (HGF), and β-nerve growth factor (β-NGF) (supporting information Fig. 2).

ASCs Persist in the Border Zone for at Least 1 Month Following Administration

At 4 weeks postimplantation, 3 of 10 hearts in the cell-treated group but none of the 10 hearts in the saline group showed evidence of persistently engrafted human ASCs, which were identified by detection of HLA-ABC antigen in the analyzed sections; the sectioning sampled the myocardium, including the infarction zone, with an overall sampling fraction of approximately ¼ of the total tissue (Fig. 5). The human cells were located exclusively at the infarct border zone (Fig. 5A, 5E). Many of the $HLA\text{-}ABC^+$ ASCs were viable and also remained in cell cycle, as indicated by positive staining for Ki67 (Fig. 5B) [24]. There was no evidence that engrafted ASCs differentiated into cardiomyocytes, as indicated by the absence

of HLA-ABC-expressing cells that were also positive for the myocyte-specific marker α-sarcomeric actin, in all examined sections (Fig. 5F).

The previous reports that adipose-derived cells can occasionally adopt endothelial phenotypes and stably engraft in host vasculature led us to examine evidence for this. Immunofluorescent analysis of sections from ischemic muscles from the treated mice indicated that SMA-expressing human ASCs were often found in a perivascular position but were never observed within the vessel wall or luminal layer proper (Fig. 6).

Discussion

Human ASCs Are Readily Available Cells Active for Myocardial Rescue

Adult stem cells hold great promise for use in tissue repair and regeneration, and early clinical data provide support for continued development as therapy for a range of medical indications. At present, there is an absence of trials that directly compare cell types with respect to their feasibility of use, safety, or therapeutic efficacy in this context. The majority of clinical trials of multipotent stem cells for treating heart disease have tested bone marrow- and blood-derived mononuclear cells, but recently, other cell types, such as bone marrow-derived stem cells (MSCs) and ASCs, have entered into clinical testing [2,25–27]. One of the issues for the therapeutic development of any of these cells will be the safety and ease of obtaining sufficient numbers. There are practical limitations and safety concerns associated with obtaining sufficient volumes of bone marrow-derived cells for transplantation [28]. Obtaining blood-derived progenitor cells is significantly less invasive, and high quantities are available following mobilization with agents such as granulocyte colony-stimulating factor or AMD3100 [28,29]. One of the potential benefits of ASCs is their ready availability from expendable adipose tissues at high yields using minimally invasive methods. Thus, ASCs may be a particularly attractive source of therapeutic cells for heart repair, providing they demonstrate efficacy at least comparable to that of cells derived from other tissues or fluids, which will require a direct comparison in relevant models.

Human ASCs Preserve Heart Function and Limit Damage Induced by Ischemic Insult

Although ASCs have recently entered into clinical trials on the basis of positive efficacy data in disease-relevant animal models, the predominant mechanisms of their function have yet to be broadly determined. Some studies have suggested that ASCs may differentiate to regenerate host tissues, whereas others, including our own prior studies, have determined that the primary (if not exclusive) effect involves paracrine support of endogenous repair mechanisms [4–6,8–10,12,13,19]. One variable among these studies is the source of the ASCs (human or animal). To address in parallel for the first time the effects and mechanism(s) of action of human ASCs in the context of myocardial injury, we studied the effects and fate of unmodified human ASCs that were administered directly to the infarcted heart of immunodeficient athymic nude rats.

Our data extend multiple reports in hind limb and myocardial ischemia models, highlighting that human ASCs have great potential for limiting the damage induced by ischemic insult. A marked stabilization of ventricular shape, along with a slight (3.4%) improvement in ejection fraction from the subacute post-myocardial infarction (post-MI) time point (4-day post-LAD occlusion), was observed in ASC-treated hearts (Table 1). This is especially significant given that the function of hearts of control animals demonstrated a decline of 14% over the same time period. This benefit was reflected by multiple functional and morphometric measures.

Post-mortem tissue analysis indicated that the functional benefit of ASCs was accompanied by significant reduction in the infarction volume observed at 28 days. Key mechanisms by which human ASCs might preserve heart function after ischemic insult include contribution

of new myocardial tissue either by direct differentiation to cardiomyocytes or by augmentation of myocardial differentiation from pre-existing resident stem or progenitor cells [30], a mechanism that has gained significant recent attention, and reduction of myocardial loss through support of ischemic but viable myocardium in the region of ischemia. Each of these putative mechanisms depend on the provision of paracrine support by the ASCs and might function to prevent expansion of the scar region.

This study indicates that the reduction of infarction was accompanied by promotion of enhanced microvessel densities at the borders of the infarct. We have previously demonstrated that ASCs secrete factors that are known to possess both angiogenic and anti-apoptotic effects on both myocardial and endothelial cells, among others [12]. Two significant and abundant paracrine factors secreted by ASCs are VEGF and HGF [10,12,13,19]. Indeed, we have previously demonstrated in vivo, via stable HGF knockdown, that this factor plays a preeminent role in ASC effects in ischemic skeletal muscle tissue [10]. HGF is a pleiotropic molecule that possesses potent prosurvival, angiogenic, and motogenic properties [31,32]. The HGF receptor (c-Met) is expressed by cardiac progenitor cells, and its expression is upregulated in mature cells within viable myocardium following acute infarction, suggesting an important signaling function of HGF during recovery from ischemic damage [33–35]. These observations suggested the hypothesis that ASCs act to directly attenuate apoptosis of myocardial cells during the acute ischemia phase, thereby preventing one of the major contributions to the development of myocardial infarcts [17,36,37].

In addition to the putative protective effects of ASCs, a complementary repair process may also occur whereby growth factors secreted by ASCs enhance homing to and subsequent differentiation and stable engraftment of resident cardiac progenitors within the injury site [35]. Subsequent stimulation of vessel growth by transplanted ASCs could further contribute to persistent tissue viability in the ischemic boundary zone by promoting angiogenesis and vascular stability (as shown by increased small arteriole density in border zone), as was observed previously in ischemic skeletal tissues [5,6,10,19,35]. The overall effect of these processes would explain the preservation of myocardial architecture and function in the ASC-treated group.

ASCs Transplantation Induces Nerve Sprouting

A new finding of these studies is that ASCs induce nerve sprouting in myocardial tissues bordering the infarct, a process that is associated with recovery of myocardial metabolic activity and reestablishment of contractile performance [38]. Our prior study has shown that mesenchymal stem cell injection into the swine myocardium can induce cardiac nerve sprouting and sympathetic hyperinnervation, although functional correlates were not evaluated in that study [39]. It is known that cardiac nerve sprouting occurs in response to MI [22], nerve growth factor infusion into stellate ganglia [40], and chronic high-frequency electrical stimulation [41]. A potential benefit of cardiac reinnervation is improved hemodynamic performance, as has been demonstrated in cardiac transplant recipients [42–44]. However, hypertrophic reinnervation is also associated with arrhythmogenesis and a high incidence of sudden cardiac death following acute MI [45,46]. The low incidence of acute deaths of rats after inducing MI in this study was the same in both groups (one animal per group), indicating that transplantation of ASCs did not exacerbate lethal arrhythmias in this model. In addition, studies in a chronic infarct swine model have suggested that ASCs are not arrhrythmogenic in that context [47].

Although the mechanism by which ASCs augment nerve sprouting has not yet been established, we have been able to detect significant NGF production by ASCs grown in culture, and indeed it is possible that conditions in vivo may further stimulate its expression. In addition, nerve growth is dependent upon both VEGF and overall vascularization, and new as well as

established nerves are invariably associated with vessels [40,41]. Thus, ASCs may directly stimulate nerve sprouting and also provide support to regenerating nerves by directing increased vascularity of tissues. Regardless of the mechanism responsible, it is intriguing to speculate that augmented reinnervation of ischemic tissues could play a complementary role in enhancing contractile performance and compensating for loss of function in infarcted regions.

Engraftment and Fate of ASCs in Infarcted Hearts

Although stable engraftment of ASCs into the heart after 1 month was clearly demonstrated in multiple hearts, we have found no evidence that the donor cells had differentiated into myocardial cells, which contrasts with at least two previous reports in ischemic myocardium models [9,48]. A previous study with murine ASCs expressing the β-galactosidase transgene demonstrated preserved heart function and persistence of ASCs in myocardium. Donor murine ASC engraftment in the myocardium was demonstrated up to 2 weeks following injection, and many of the ASCs expressed cardiomyocyte markers. A subsequent study in a rat chronic infarction model demonstrated improved heart function and persistence of rat ASCs that were marked with the fluorescent nuclear marker DAPI [48]. In the latter study, the transplanted ASCs persisted for up to 4 weeks and demonstrated certain endothelial properties.

Our study clearly demonstrated localization of identified ASCs near viable myocardium, which supports the hypothesis of a supportive paracrine mechanism by which ASCs promote myocardial tissue survival, neovascularization, or even recruitment or activation of endogenous cardiac stem cells. This is also consistent with our recent studies in ischemic skeletal tissues demonstrating a preeminent paracrine function of human donor ASCs [10].

It is possible that the inconsistency with previous results demonstrating that ASCs productively engraft into host cardiac tissues may be attributed to differences in cell isolation or culture methods, or indeed may be specific to the use of human cells in a xenogeneic setting. However, the fact that a significant response was observed upon cell administration, in the absence of widespread direct tissue replacement by ASCs, indicates that trophic factor secretion is an important mechanism by which ASCs promote repair of ischemic heart tissues. Meanwhile, it must be recognized that a potential limitation of the study is that only a portion (approximately 30%) of all the myocardial tissue was analyzed by histology; accordingly, low-frequency myocardial fate of ASCs cannot be ruled out. Nevertheless, low-frequency engraftment would be difficult to reconcile with a notion that such differentiation contributed significantly to the observed functional gains.

Conclusion

The present study shows that local myocardial delivery of ASCs at the time of MI leads to a sustained improvement in cardiac function and reduction of adverse myocardial remodeling. A substantial portion of the effects of ASCs are likely related to paracrine support of ischemic myocardium, rather than cardiomyogenic differentiation of the donor cells. Our findings suggest that ASCs and factors secreted by these cells may have important therapeutic application for cellular therapy in prevention of ischemic tissue damage. Although the effects of ASCs have not been directly compared with those of other cell types, the ready and practical availability of ASCs in clinical practice supports the notion that therapies with these cells may have a high potential for widespread clinical adoption, provided that the functional outcomes demonstrated in this study can be extended to the human context.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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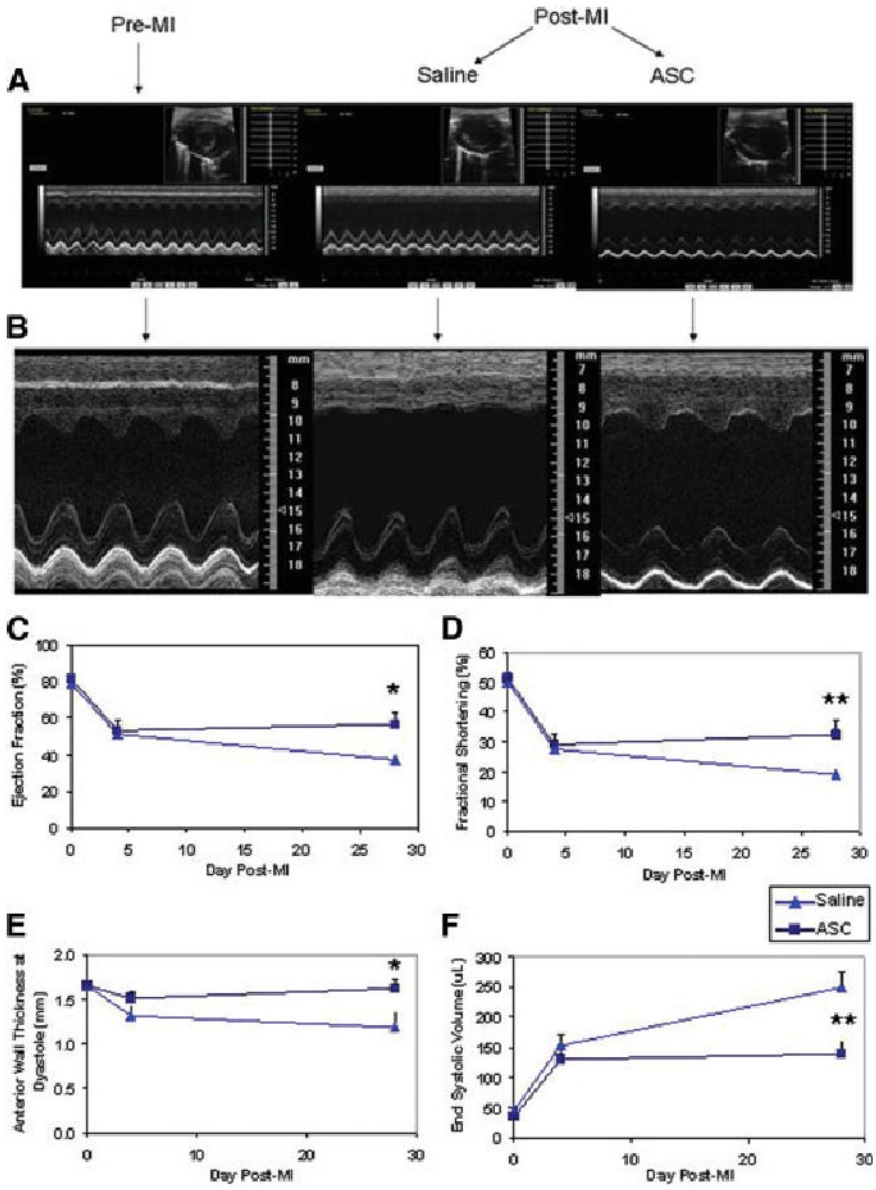

Figure 1.

Serial echocardiographic analysis of heart function demonstrates a functional improvement due to ASC treatment. (**A**): Examples of two-dimensional (2D) and M-mode echocardiographic images obtained from a normal heart before infarction (pre-MI) and then again at 28 days after MI and injections with either saline or ASCs. An evident deficit in the anterior wall motion (top of each image) was detected, which was partially ameliorated by ASC treatment. (**B**): Enlarged M-mode images shown in (**A**). (**C**–**F**): Graphs of functional parameters obtained from echocardiographic images (mean ± SEM; $n = 10$ for each value). Parameters shown are ejection fraction as determined from 2D images (**C**), fractional shortening as determined from 2D images (**D**), anterior wall thickness as determined from M-mode images (**E**), and end-systolic volume as determined from 2D images (**F**). A complete listing of parameters is shown in Table 1. *, $p < .05$; **, $p < .01$. Abbreviations: ASC, adipose-derived stromal/stem cell; MI, myocardial infarction.

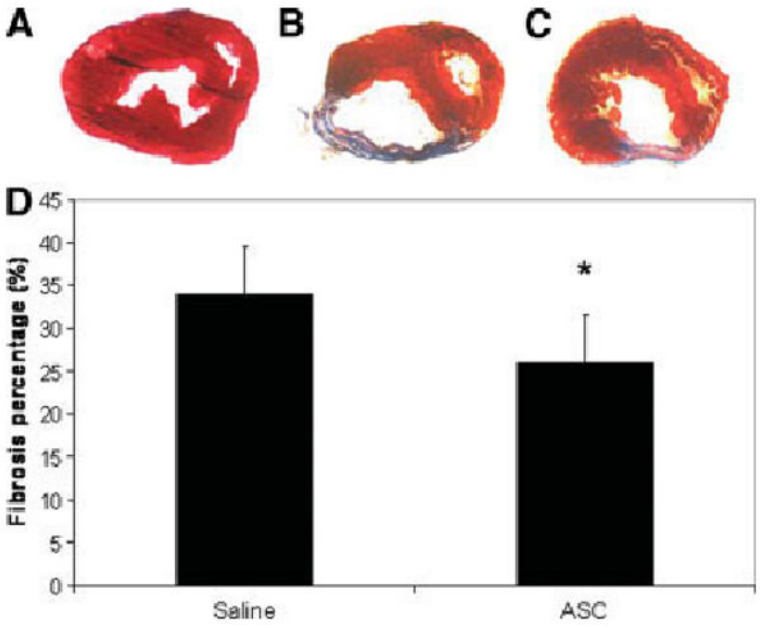

Figure 2.

Injection of acutely infarcted hearts with ASCs attenuated the extent of infarction. Images of thin sections of a normal heart (**A**) or hearts at 28 days after infarction and treatment with either saline (**B**) or ASCs (**C**). Red indicates viable myocardium; blue indicates fibrosis due to infarction damage. The percentage of infarcted left ventricular area was determined by planimetry, and the average ± SEM for all hearts ($n = 10$) in the two groups is shown (**D**). The infarction area was reduced in ASC-treated hearts (26% ± 6% vs. 34% ± 6% in saline) (*, $p < .05$). Abbreviation: ASC, adipose-derived stromal/stem cell.

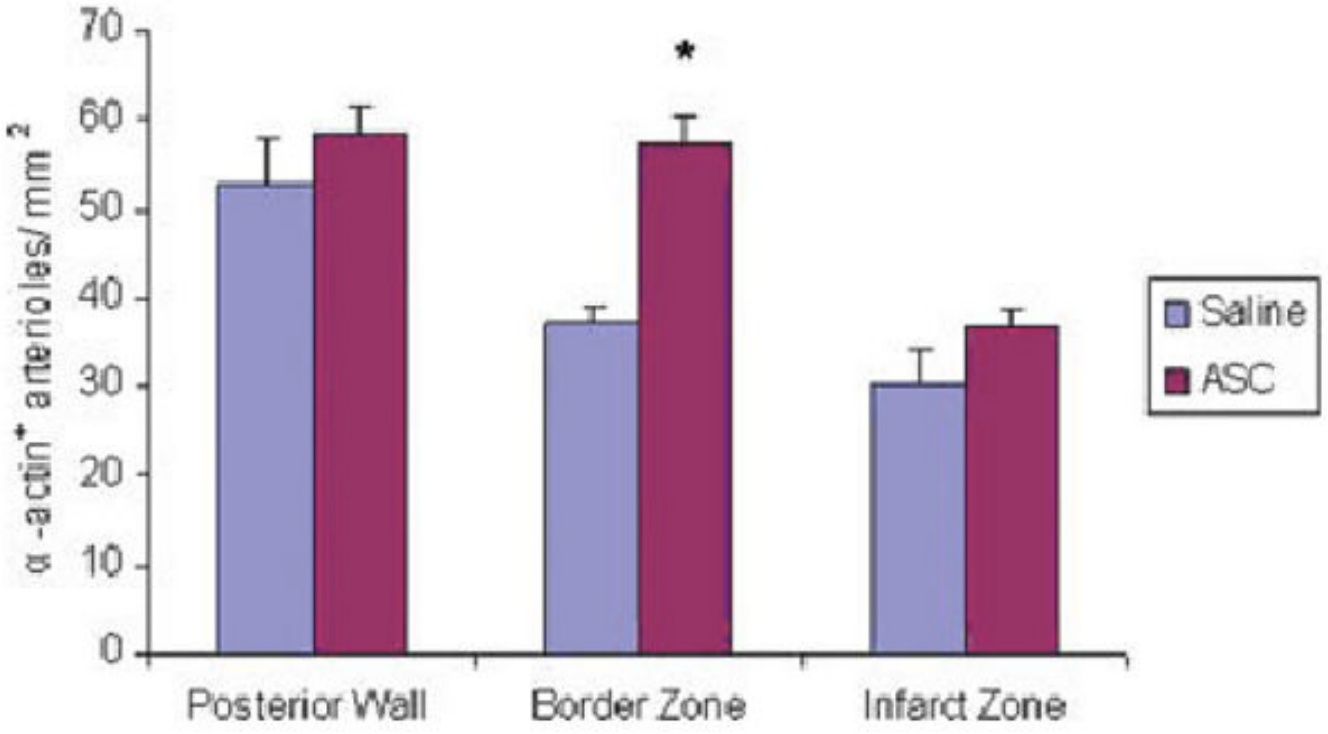

Figure 3.

Microvessel densities within the border zone of the infarct were enhanced by treatment with ASCs. Immunohistochemical analysis of thin sections from each heart ($n = 10$ for each group) was performed with antibodies to smooth muscle α-actin (SMA). The density of smooth muscle SMA^+ arterioles was determined in the remote posterior regions, as well as border and infarct zones, as described in Materials and Methods. The number of microvessels per area was greater in ASC-treated hearts than in the saline control group (*, $p < .05$). Abbreviation: ASC, adipose-derived stromal/stem cell.

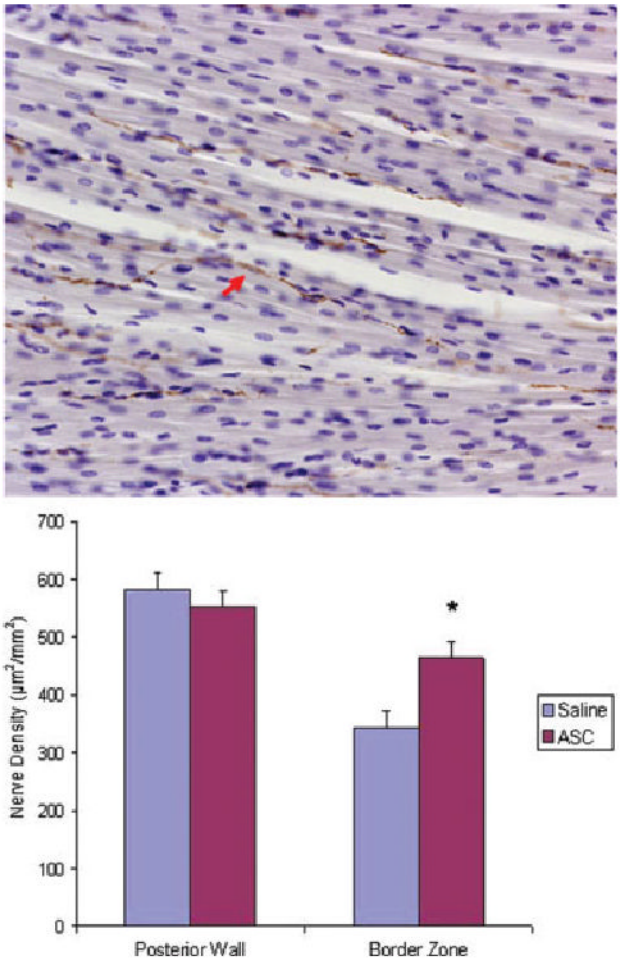

Figure 4.

Treatment with ASCs promotes enhanced nerve sprouting in viable peri-infarction regions of the heart. (**A**): Representative photomicrograph of growth-associated protein-43 $(GAP43)^+$ nerve sprouts (red arrow) in the left ventricular region 1 month after infarction and treatment with ASCs. (**B**): ASCs induced more $GAP43^+$ nerve sprouts in the border zones of the infarct ($464 \pm 43\ \mu m^2/mm^2$) compared with the control group ($342 \pm 20\ \mu m^2/mm^2$) (*, $p < .05$). There was no significant difference between the two groups in the posterior myocardial wall nerve sprouting. Abbreviation: ASC, adipose-derived stromal/stem cell.

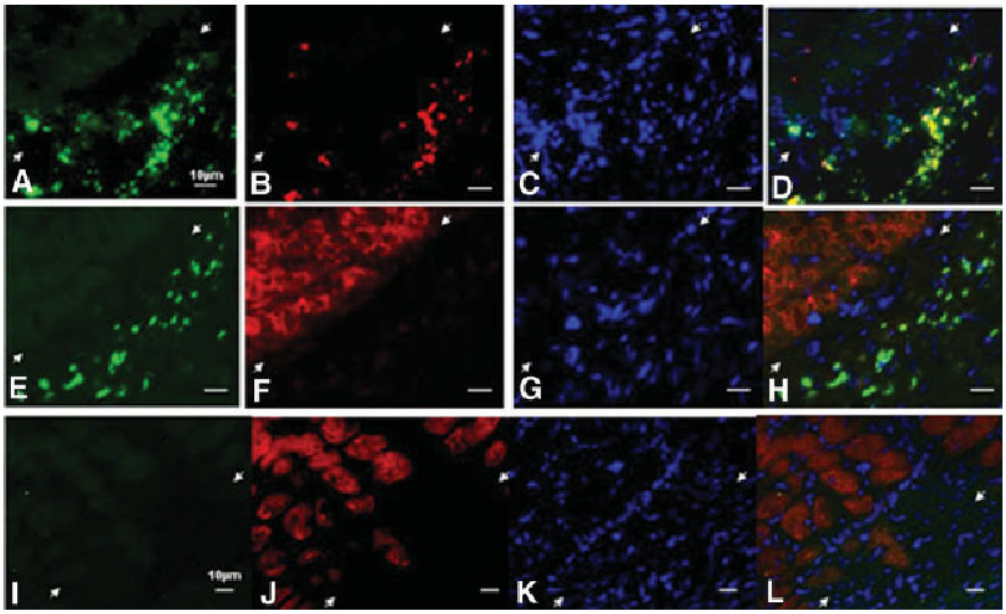

Figure 5.

Viable adipose tissue-derived stromal/stem cells (ASCs) stably engraft in the regions adjacent to, but distinct from, viable cardiomyocytes. (**A**): Immunofluorescent detection of donor ASCs was accomplished by probing for human leukocyte antigen (HLA)-ABC (green). The ASCs were located within the infarct region (white arrows in all panels demarcate the border zone). (**B**): These ASCs were viable and actively proliferating as evidenced by the expression of the Ki67 marker (red). (**C**): Visualization of nuclei with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) stain. (**D**): Merged images shown in (**A–C**) demonstrate colocalization of Ki67 and HLA-ABC staining (yellow). (**E–H**): Clusters of ASCs were often observed in proximity to live myocardium within peri-infarct regions. (**E**): Immunofluorescent images of HLA-ABC-expressing ASCs (green). (**F**, **G**): Viable α-sarcomeric actin^{+} cardiomyocytes (red staining in (**F**)) and DAPI-stained nuclei (blue staining in (**G**)). (**H**): The merged images (**E–G**) provides evidence that ASCs reside in juxtaposition with cardiomyocytes and are not present within viable myocardium. (**I**): Probing similar sections with an isotype control antibody demonstrated the specificity of the HLA-ABC antibody for human-derived ASCs. (**J**, **K**): Costaining of the section shown in (**I**) with an antibody to α-sarcomeric actin and DAPI nuclear stain. (**L**): Merged images (**I–K**). Scale bars = 10 μm.

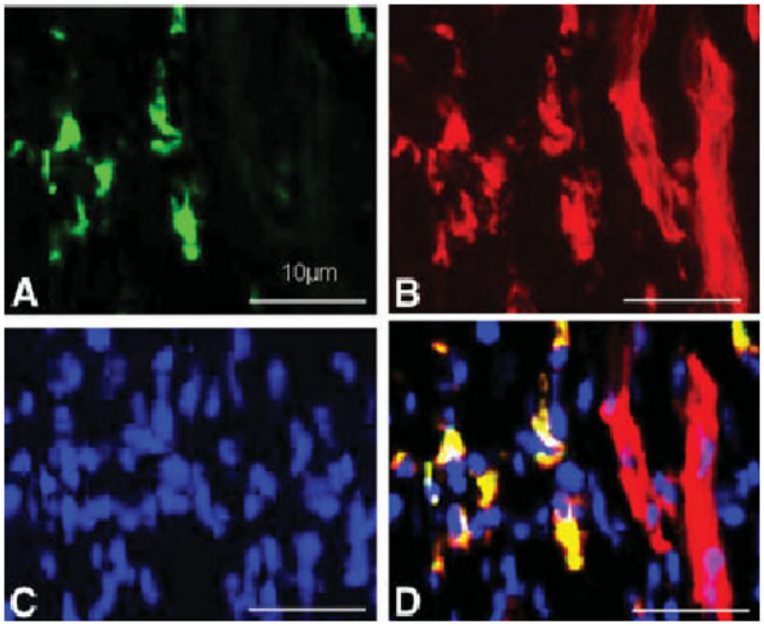

Figure 6.

Long-term engraftment of adipose tissue-derived stromal/ stem cells (ASCs) occurs in proximity to vascular structures. **(A–D)**: Immunofluorescent micrographs of a thin section from an infarcted rat heart at 1 month after treatment with ASCs. **(A)**: Detection of human leukocyte antigen (HLA)-ABC^{+} ASCs (green). **(B, C)**: The same section probed with antibodies to smooth muscle α-actin (SMA) (red) **(B)** and 4′,6-diamidino-2-phenylindole dihydrochloride nuclear stain (blue) **(C)**. **(D)**: Merged images **(A–C)** show the coexpression of HLA-ABC and SMA antigens by the ASCs. A branching vessel is evident in the right portion of the image. Scale bars = 10 μm.

Table 1

Serial echocardiographic analysis of functional and morphometric parameters of hearts from saline control and ASC treatment groups

		Saline control (n = 10)			ASC treatment (n = 10)			
Parameter	**Preinfarction**	**Day 4**	**Day 28**	***p* value 4 vs. 28**	**Day 4**	**Day 28**	***p* value 4 vs. 28**	***p* value day 28 saline vs. ASC**
LVEDV (mm^3)	200 + 13	309 + 23	391 + 30	<.05	263 + 25	311 + 17	NS	<.05
LVESV (mm^3)	38.5 ± 3.6	152 ± 18	249 ± 27	<.001	129 ± 22	139 ± 21	NS	<.01
LVEDD (mm)	6.5 + 0.2	7.6 + 0.2	8.7 + 0.2	<.001	7.2 + 0.3	7.6 + 0.2	NS	<.01
LVESD (mm)	3.3 ± 0.1	5.6 ± 0.4	6.6 ± 0.5	<.05	5.3 ± 0.4	5.2 ± 0.4	NS	<.05
Ant wall diast (mm)	1.7 ± 0.1	1.3 ± 0.1	1.2 ± 0.2	NS	1.5 ± 0.1	1.6 ± 0.1	NS	<.05
Ant wall syst (mm)	2.9 ± 0.1	2.9 ± 0.1	2.0 ± 0.3	NS	2.2 ± 0.2	2.5 ± 0.2	NS	<.05
Post wall diast (mm)	1.8 ± 0.1	1.7 ± 0.1	1.8 ± 0.3	NS	1.9 ± 0.2	2.1 ± 0.2	NS	NS
Post wall syst (mm)	2.7 ± 0.1	2.3 ± 0.2	2.4 ± 0.2	NS	2.5 ± 0.1	2.7 ±0.1	NS	NS
EF (%)	81.1 ± 0.9	51.4 ± 4.5	37.2 ± 3.0	<.05	53.2 ± 5.3	56.6 ± 6.8	NS	<.05
FS (%)	50.9 + 1.0	27.7 + 3.0	18.9 + 1.7	<.05	29.2 + 3.6	32.5 + 4.7	NS	<.01

Statistical analysis of the difference within groups at days 4 and 28 was performed using a two-tailed, paired Student's *t* test. Between-group comparisons were made with a two-tailed, unpaired Student's *t* test.

Abbreviations: ant wall diast, anterior wall thickness in diastole; ant wall syst, anterior wall thickness in systole; ASC, adipose tissue-derived stromal/stem cell; EF, ejection fraction; FS, fractional shortening in left ventricular diameter; LVEDD, left ventricular end-diastolic diameter; LVEDV, left ventricular end-diastolic volume; LVESD, left ventricular end-systolic diameter; LVESV, left ventricular end-systolic volume; NS, not significant; post wall diast, posterior wall thickness in diastole; post wall syst, posterior wall thickness in systole.