Cell Transplantation, Vol. 16, pp. 879–886, 2007
Printed in the USA.

0963-6897/07 $90.00 + .00
E-ISSN 1555-3892
www.cognizantcommunication.com

SDF-1 Recruits Cardiac Stem Cell-Like Cells That Depolarize In Vivo

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Prolongation or reestablishment of stem cell homing through the expression of SDF-1 in the myocardium has been shown to lead to homing of endothelial progenitor cells to the infarct zone with a subsequent increase in vascular density and cardiac function. While the increase in vascular density is important, there could clearly be other mechanisms involved. In a recent study we demonstrated that the infusion of mesenchymal stem cells (MSC) and MSC that were engineered to overexpress SDF-1 led to significant decreases in cardiac myocyte apoptosis and increases in vascular density and cardiac function compared to control. In that study there was no evidence of cardiac regeneration from either endogenous stem cells or the infused mesenchymal stem cells. In this study we performed further detailed immunohistochemistry on these tissues and demonstrate that the overexpression of SDF-1 in the newly infracted myocardium led to recruitment of small cardiac myosin-expressing cells that had proliferated within 2 weeks of acute MI. These cells did not differentiate into mature cardiac myocytes, at least by 5 weeks after acute MI. However, based on optical mapping studies, these cells appear capable of depolarizing. We observed greater optical action potential amplitude in the infarct border in those animals that received SDF-1 overexpressing MSC than observed in noninfarcted animals and those that received control MSC. Further immunohistochemistry revealed that these proliferated cardiac myosin-positive cells did not express connexin 43, but did express connexin 45. In summary, our study suggests that the prolongation of SDF-1 expression at the time of acute MI leads to the recruitment of endogenous cardiac myosin stem cells that may represent cardiac stem cells. These cells are capable of depolarizing and thus may contribute to increased contractile function even in the absence of maturation into a mature cardiac myocyte.

Key words: SDF-1 expression; Cardiac stem cells; Depolarization; Cardiac myocytes

INTRODUCTION

Since the advent of reperfusion therapy for the treatment of acute myocardial infarction (AMI) there has been a progressive decrease in the mortality rate of patients who experience AMI, with mortality rates in clinical trials decreasing from greater than 15% in clinical trials in the early 1980s to less than 5% in trials in the current era of primary percutaneous coronary intervention (19). This success alone has led to an increasing burden of patients with ischemic cardiomyopathy and chronic heart failure (CHF). In an attempt to develop novel strategies to prevent or treat CHF many groups have been investigating the utility of cell transplantation or cell-based gene therapy (17) as a means of either potentially regenerating myocardial tissue (15,21,25) or modulating the pathological remodeling that occurs following myocardial cell death (1,5,6,9,22).

Early studies suggested that the transplantation of multiple different stem cell populations could lead to regeneration of myocardial tissue, including cardiac myocytes, endothelial cells, and smooth muscle cells (15,16, 21). While more recent studies have verified the ability of stem cell transplantation to improve cardiac function, they have failed to validate the presence of cardiac myocyte regeneration (3,13,24). Thus, while there is significant excitement at the potential for cell-mediated repair of the myocardium, the goal of myocardial regeneration still appears elusive, and ultimately may await the development of embryonic stem cell (10) or adult-derived embryonic-like stem cell-based (8) therapies.

We have previously identified stromal cell derived

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factor-1 (SDF-1) as a myocardial stem homing factor (2,7). CXCR4 is the receptor for SDF-1 and is expressed on hematopoietic stem cells and their progeny endothelial progenitor cells (EPC). SDF-1 expression is temporally limited following AMI. We have previously hyopothesized that the short-term expression of SDF-1 following AMI could limit myocardial healing. The reestablishment of SDF-1 expressing at times remote from AMI results in recruitment of EPC (2,23), increased vascular density, and improvement in myocardial function (2,7). In our earlier studies we proposed that the improvement observed in myocardial function was due to the increase in vascular density through the recruitment of EPC. While the increase in vascular density may be important, recent studies have suggested that there are endogenous stem cells in the heart. At least one group has suggested that these cells are CXCR4 positive. We have recently demonstrated that the overexpression of SDF-1 in myocardial tissue through the infusion of SDF-1-overexpressing mesenchymal stem cells results in significant preservation, but not regeneration, of mature cardiac myocytes at least at 5 weeks after AMI. The goal of this study is to determine if there is evidence of mobilization of cardiac stem cell-like cells in the infarct border zone in animals that receive MSC or SDF-1-overexpressing MSC, and whether these cells may be electrically active despite not differentiating into mature cardiac myocytes.

MATERIALS AND METHODS

Myocardial Infarction

All animal protocols were approved by the Animal Research Committee and all animals were housed in the AAALAC animal facility of the Cleveland Clinic. Ligation of the left anterior descending artery in Lewis rats was performed as previously described (24). Animals were anesthetized with IP ketamine and xylazine, intubated, and ventilated with room air at 75 breaths per minute using a rodent ventilator (RSP1002, Kent Scientific Corp, Torrington, CT). The anterior wall myocardial infarction was produced by ligating the left anterior descending (LAD) artery with using a surgical microscope (M500, Leica™ Microsystems, Bannockburn, IL). The tissue sections analyzed in this study were from the animals generated for our recent study (24).

Cell Preparation and Delivery

Rat bone marrow was isolated by flushing Lewis rat femurs with 0.6 ml DMEM (GIBCO, Invitrogen, Carlsbad, CA). Clumps of bone marrow were gently minced with a 20-gauge needle. Cells were separated by Percoll density gradient. The cells were centrifuged for 10 min at $260 \times g$ and washed with three changes of PBS with 100 U/ml penicillin, 100 g/ml streptomycin (Invitrogen, Carlsbad, CA). The washed cells were then resuspended and plated in DMEM-LG (GIBCO) with 10% FBS and 1% antibiotic and antimycotic solutions (GIBCO). The cells were then incubated at 37°C. Nonadherent cells were removed by replacing the medium after 3 days. Cultures were refed every 3–4 days. Once cultures became 70% confluent, adherent cells were detached following incubation with 0.05% trypsin and 2 mM EDTA (Invitrogen) for 5 min and subsequently passaged. Preceding experiments utilizing MSC, the cultures were simultaneously depleted of $CD45^+$, $CD34^+$ cells by negative selection using 10 μl/10^6 cells of each of the following primary PE-conjugated antibodies: mouse anti-rat CD45 (BD Biosciences, San Diego, CA) and mouse anti-CD34 antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). PE-positive cells were negatively selected using the EasySep PE selection kit according to the manufacturer's instructions (Stem Cell Technologies). Confluent cells were passaged and plated out at 1:2 to 1:3 dilutions until passage 11. Cells were assayed for their ability to differentiate into the adipogenic, chondrogenic, and osteogenic lineages. Cells were maintained in differentiation media for 2–3 weeks. Differentiation was validated by staining the cells with Oil Red (adipogenic lineage), alcian blue (chondrogenic lineage), or alkaline phosphatase (osteogenic lineage). Cells were induced to overexpress SDF-1 by transfection with a plasmid construct that encoded SDF-1 and antibiotic selection to select for a population of cells that was stably transfected cells. Two million labeled cells (MSC or SDF-1-expressing MSC) suspended in 200 ml of PBS or 200 ml of PBS alone were infused via tail vein injection 24 h after myocardial infarction.

BrdU and GFP Labeling

BrdU (50 mg/kg) was injected IP every 12 h for 14 days beginning the day after cell transplantation. With respect to the GFP, we used a VSV-G pseudotyped lentivirus expressing EGFP with a nuclear localizing sequence or SDF-1 driven by the CMV promoter. The lentivirus was made using four plasmid vector system by the Viral Core at the Cleveland Clinic. The MSC were transduced twice for 8 h with purified lentivirus in the presence of 8 μg/ml of polybrene at a multiplicity of infection (MOI) of 30. The medium was changed 72 h posttransfection and replaced with regular medium containing zeocin (EGFP) or zeocin and blasticidin (hSDF1 and EGFP). Thus, only cells that incorporated the viral genome, including the zeocin and/or blasticidin resistance gene, survived.

Immunostaining

Animals were sacrificed 5 weeks following myocardial infarction. Tissues were fixed in formalin and em-

bedded in paraffin blocks according to established protocols. Antigen retrieval was performed using 10 mM sodium citrate buffer (pH 6.0) and heat at 95°C for 5 min. The buffer was replaced with fresh buffer and reheated for an additional 5 min and then cooled for approximately 20 min. The slides were then washed in deionized water three times for 2 min each. Specimens were then incubated with 1% normal blocking serum in PBS for 60 min to suppress nonspecific binding of IgG. Slides were then incubated for 60 min with the primary antibody. Optimal antibody concentration was determined by titration. Slides were then washed with PBS and incubated for 45 min, with secondary antibody diluted in PBS with serum, in a dark chamber. After washing extensively with PBS, coverslips were mounted with aqueous mounting medium (Vectashield Mounting Medium with DAPI, H-1200; Vector Laboratories, Burlingame, CA).

Confocal Immunofluorescence Microscopy

Tissue was analyzed using a upright spectral laser scanning confocal microscope (Model TCS-SP; Leica Microsystems, Heidelberg, Germany) equipped with blue argon (for DAPI), green argon (for Alexa Fluor 488), and red krypton (for Alexa Fluor 594) laser. Data were collected by sequential excitation to minimize "bleed-through." Image processing, analysis, and the extent of colocalization were evaluated using the Leica Confocal software. Optical sectioning was averaged over four frames and the image size was set at 1024×1024 pixels. There were no digital adjustments made to the images.

Antibodies Implemented in These Studies

Antibodies used were: mouse anti-myosin ventricular heavy chain α/β monoclonal antibody (Chemicon International, Inc.); rabbit anticonnexin-43 polyclonal IgG antibody (Santa Cruz Biotechnology, Inc.); rabbit anticonnexin-45 polyclonal IgG antibody (Santa Cruz Biotechnology, Inc); sheep polyclonal to BrdU biotin (abcam) and mouse monoclonal anti BrdU (Roche).

Optical Mapping

Optical mapping as previously described (11,12) using Langendorff-perfused rat hearts was done 1 month after MI. ECG, perfusion pressure, and bath temperature were measured continuously during all experiments. The optical mapping system used has been described in detail previously. Briefly, action potentials were optically recorded at a magnification of 2.1?× from sites within an 8.3×8.3-mm mapping field (0.52-mm interpixel resolution) on the anterior epicardial surface of the left and right ventricle. Fluorescence was excited with uniform light from a 270-W tungsten-halogen light source (filtered 514 + 20 nm) and transmitted to a 16×16-element photodiode array detector through a tandem-lens imaging system (emission filter >610 nm). Photocurrent from each photodiode underwent current-to-voltage conversion, amplification, and bandpass filtering (0.1–500 Hz) and was multiplexed and digitized (1000 samples/s per channel) with 12-bit precision. A CCD camera that is optically aligned with the photodiode array was used to obtain visible images of the optical mapping field relative to anatomical landmarks (e.g., infarct scar and border zone).

RESULTS

SDF-1 Recruitment of Cardiac Stem Cell-Like Cells

Figure 1 describes the experimental plan we implemented in these studies. The functional effects and histological parameters unrelated to the goals of this study have been published elsewhere (24). Briefly, we engineered mesenchymal stem cells (MSC) to overexpress SDF-1 and infused them via the tail vein of Lewis rats 1 day after LAD ligation. The overexpression of SDF-1 led to approximately threefold increase in SDF-1 secretion by the MSC over 24 h. We observed a significant increase in the number of cardiac myocytes within the infarct zone and cardiac function 5 weeks later in those animals that received SDF-1-overexpressing MSC compared to control MSC or saline (at 5 weeks: saline vs. MSC vs. SDF-1/MSC fractional shortening: $10.9 \pm 1.0\%$ vs. $17.6 \pm 3.0\%$ vs. $34.9 \pm 8.5\%$) (24). The increase in the number of cardiac myocytes was not due to MSC differentiation into cardiac myocytes because the MSC were genetically labeled to express GFP and the cardiac myocytes were not GFP positive.

In order to determine if the cardiac myocytes were derived from endogenous stem cells we repeated our studies and administered BrdU to the animals for 14 days following AMI, under the hypothesis that endogenous stem cells would proliferate at some point during the homing process and before differentiating into cardiac myocytes. We observed no BrdU-positive cardiac myocytes, making the regeneration of cardiac myocytes from endogenous stem cells unlikely (24).

Figure 2 depicts representative images from the infarct border zone 5 weeks after AMI in animals that received saline, 2 million MSC, or 2 million SDF-1-overexpressing MSC. These images demonstrate that we observed a significant increase in the number of BrdU-positive cells in those animals that received MSC compared to saline-treated animals, with the greatest number of BrdU-positive cells being found in the MSC/SDF-1 group.

While no mature cardiac myocytes were BrdU positive, we did observe that many of the the BrdU-positive cells were cardiac myosin positive (Fig. 2, yellow cells,

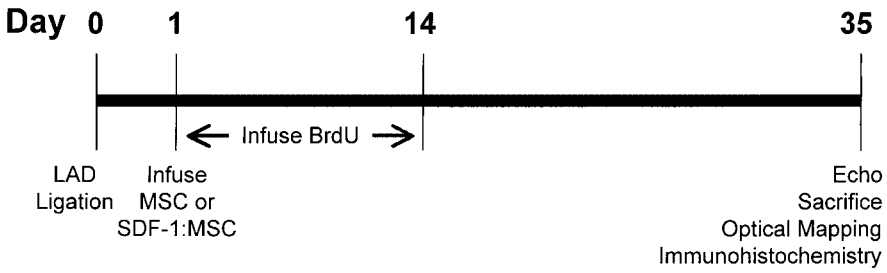

Figure 1. Schematic representation of the experimental plan implemented to study the effects of prolongation of SDF-1 expression following AMI.

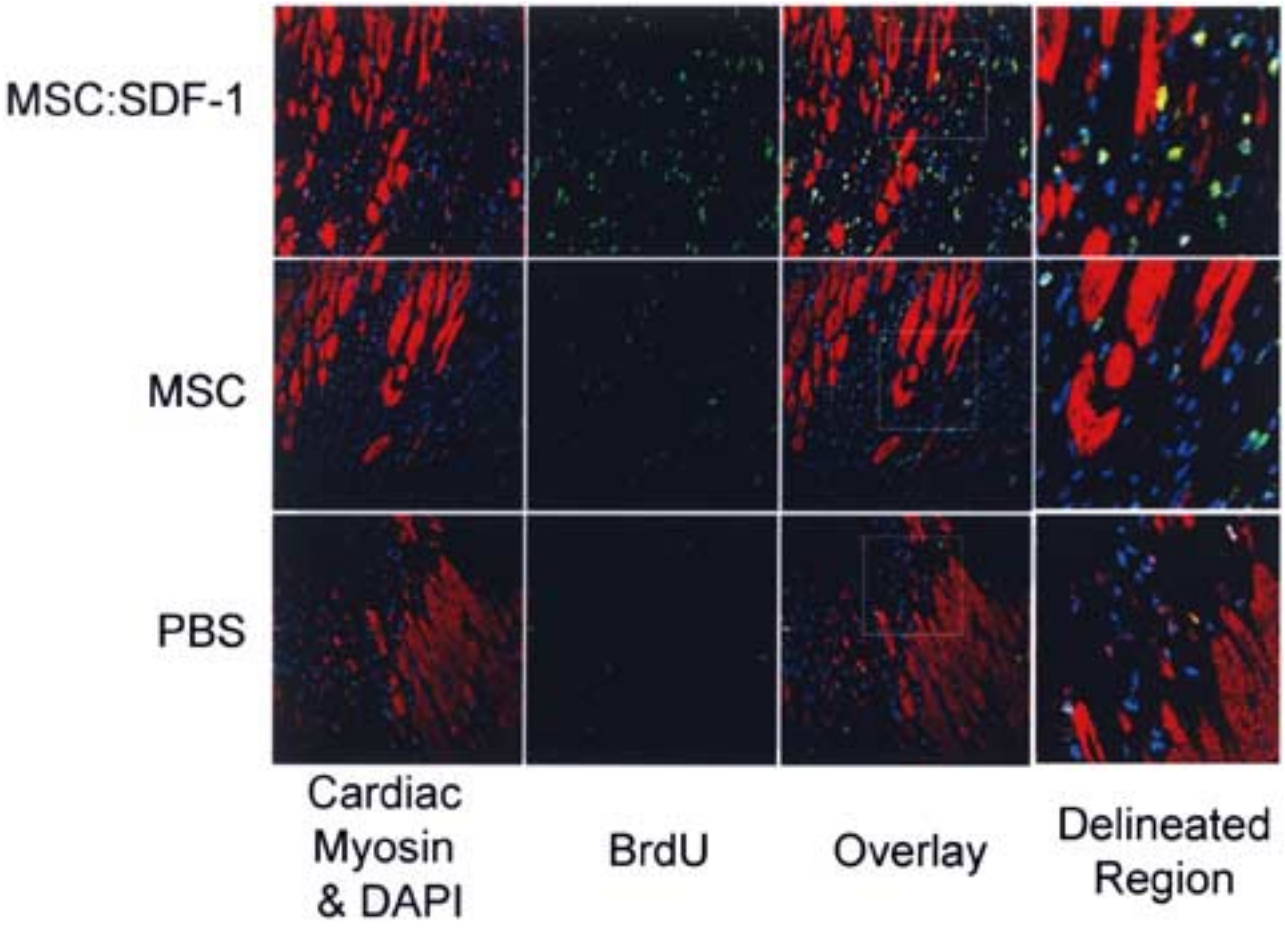

Figure 2. All representative images are from tissue 5 weeks after AMI and infusion of 2 million of control or SDF-1-expressing GFP-positive MSC 1 day after AMI. All animals received BrdU twice daily for 14 days beginning on the day after cell transplantation. Confocal images of immunofluorescent staining in the infarct border zone for cardiac myosin (red), BrdU (green), and cell nuclei (DAPI, blue) from animals that received PBS or control or SDF-1-expressing MSC. Column of images on the right are high-power images of the delineated areas in the low-power overlay images.

far right panels). While we cannot rule out that these cells were ultimately derived from the bone marrow, their proliferative capacity, their size, and their expression of cardiac myosin is consistent with them being cardiac stem cells. We quantified the number of BrdU- and cardiac myosin-positive cells per high-power field in the infarct border zone and found a significant increase in both MSC-treated groups, with an approximately four- to fivefold increase in those animals that received SDF-1-overexpressing MSC compared to control MSC (Fig. 3).

In order to further determine the etiology of the BrdU-positive cells we performed double immunostaining for BrdU and GFP to determine if the BrdU-positive cells were also from infused MSC that proliferated in vivo. That analysis revealed that in the MSC and MSC/SDF-1 groups ~20% of the BrdU-positive cells were GFP positive, suggesting that that majority of cells were not derived from the exogenous MSC (data not shown).

To further characterize the myocardial tissue following the delivery of SDF-1-overexpressing MSC we performed optical mapping. Optical action potentials were recorded from three different zones of the myocardium (infract, border zone, and noninfarct) in order to determine the effect of MSC and SDF-1-overexpressing MSC on myocardial electrical activity (Fig. 4a). We have previously shown in normal tissue that there is uniform impulse propagation and normal action potential morphology, in untreated infarcted hearts there is a rapid loss of conduction into the infarct zone, and in hearts from animals in which MSC were infused there is propagation of electrical activity into the infarct zone (12). Data from these groups are included in Figure 4 for comparison. In the MSC/SDF-1 group we observed a statistically significant increase in the electrical activity greater than that observed in normal tissue. Because cellular depolarization is a stochastic event (i.e., cells cannot partially depolarize), the only way in which we could observe an increase in action potential greater than that observed in neighboring normal or noninfarcted tissue is if there was an increase in the number of depolarizing cells in the tissue of interest.

This finding suggested that the cardiac stem cell-like cells we observed in the MSC/SDF-1 group may express

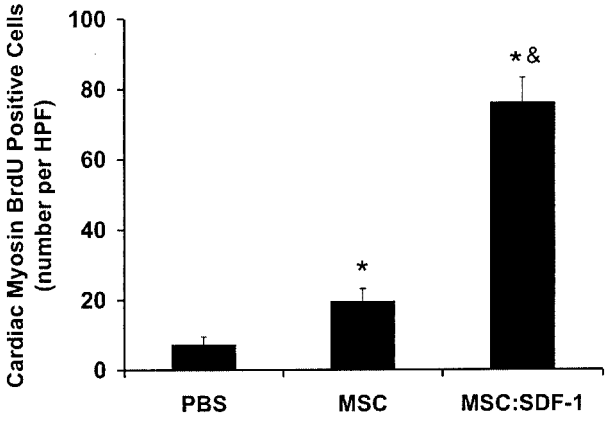

Figure 3. Number of BrdU- and cardiac myosin-positive cells per high-power field (HPF). The number of cells was quantified by two observers blinded to treatment in 5 fields per animal and 4-5 animals per treatment group. Data represent mean ± SD. *$p < 0.01$ compared to PBS; &$p < 0.0001$ compared to control MSC.

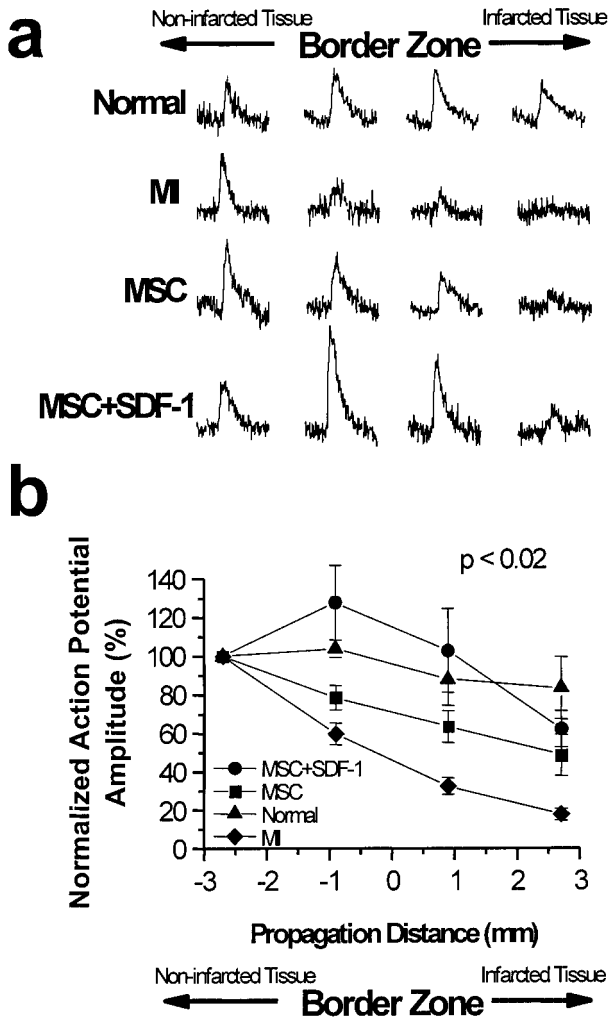

Figure 4. (a) Optically recorded action potentials obtained from normal, AMI + saline, AMI + MSC, and AMI + MSC/SDF-1 hearts as a function of distance across the border zone obtained during optical mapping. (b) Normalized magnitude of the action potential quantified as a function of distance across the infarct border zone. Magnitude of the action potential in the normal tissue prior to the infarct zone was defined as 100%. Data represent mean ± SD. $p < 0.02$ for comparison of normal tissue to MSC/SDF-1 in the proximal infarct border zone.

connexin proteins. Therefore, to further characterize the cells recruited to the infarct zone following the overexpression of SDF-1, we performed double immunofluorescence to determine if the BrdU-positive cells expressed connexin 43 or 45. They were rarely positive for connexin 43; however, they were connexin 45 positive (Fig. 5), suggesting that these cells were capable of depolarizing.

DISCUSSION

We have previously demonstrated that the reestablishment of stem cell homing at a time remote from AMI through the delivery of SDF-1-expressing cells leads to improvement in ventricular remodeling, neovascularization of the infarct zone, and increased cardiac function (2,7). More recently we have demonstrated that the prolongation of SDF-1 expression at the time of AMI leads to preservation of cardiac myocytes through the inhibition of cardiac myocyte apoptosis and neovascularization, leading to significant increases in cardiac function. While the mechanisms of these observed benefits are being elucidated, they are not fully understood.

Importantly, we have little evidence that the improvements are due to cardiac myocyte regeneration. We and others have been unable to demonstrate cardiac myocyte regeneration from mesenchymal stem cells (24), recruited CD117$^+$, or hematopoietic stem cells (2,3,7,13, 20). In our recent study (24) and this study we have been unable to demonstrate that endogenous stem cells are responsible for the increase in cardiac myocyte number following the prolongation of SDF-1 expression after AMI. However, in this study we have demonstrated that the prolongation of stem cell homing in the myocardial tissue following AMI leads to the recruitment of a small cardiac myosin-positive cell type to the infarct border zone that is consistent with cardiac stem cells (4,14). Some reports have suggested that cardiac stem cells are CXCR4 positive (4), which would be consistent with them being recruited by the prolongation of SDF-1 expression following AMI (Fig. 3). At least at 5 weeks following AMI these cardiac stem cell-like cells do not mature into mature cardiac myocytes.

While these interesting cells do not differentiate into mature cardiac myocytes at least by 5 weeks after AMI, our data demonstrate for the first time that these cells may depolarize in vivo, and thus may contribute to propagation of electrical impulses and recruitment of contractile tissue. Using the optical mapping system that we have implemented in our studies, the action potential amplitude is determined by the number of viable cells in the unit volume being measured. Therefore, for us to observe an increase in the action potential amplitude in the infarct border zone greater than neighboring normal tissue there may be an increase in the number of cells capable of depolarizing. We have previously demonstrated the importance of the expression of connexin 43 and/or 45 by cells recruited to or transplanted into the infarct zone (7,12). That the BrdU cardiac myosin-positive cells express connexin 45 thus may explain the in-

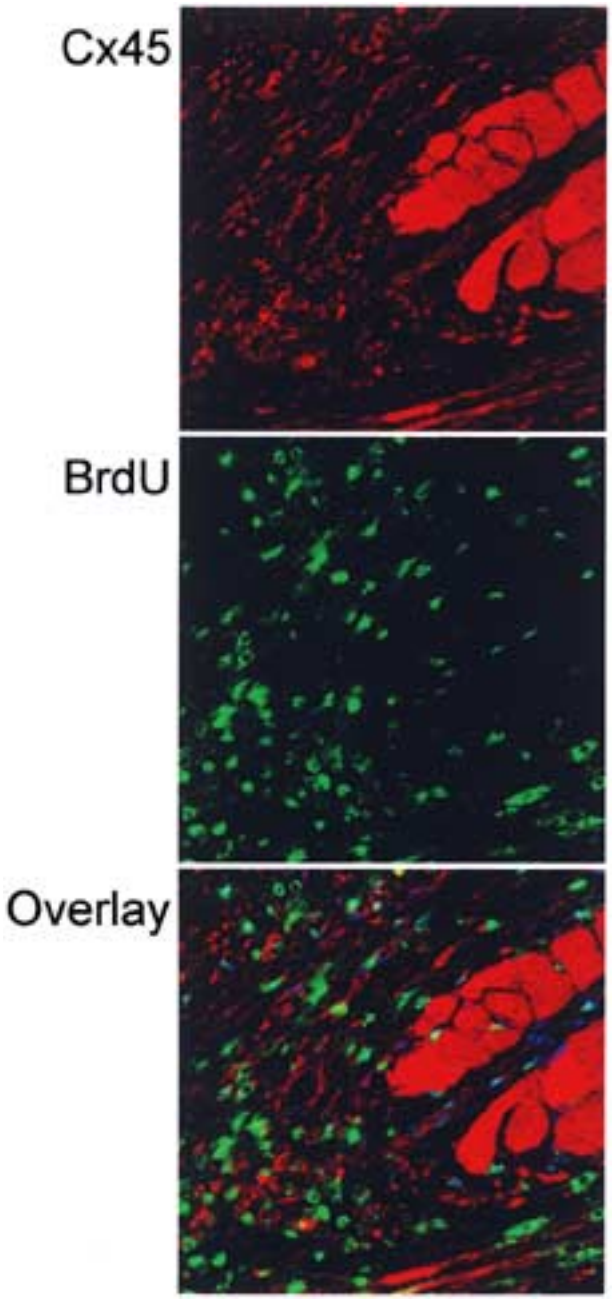

Figure 5. Representative confocal images of tissue following immunohistochemistry for BrdU (green), connexin 45 (red), and DAPI (blue).

crease in electrical activity we observed following the prolongation of SDF-1 expression.

In summary, we demonstrate for the first time that cardiac stem cell-like cells are capable of depolarizing in vivo. These results support our hypothesis (18) that there is a natural but inefficient stem cell-based repair process that occurs following AMI, and that if we can manipulate the expression of key molecular pathways, such as stem cell homing, we can significantly impact the electrical and mechanical functions of the surviving myocardium.

ACKNOWLEDGMENTS: This work was funded by NHLBI 1RO1-HL74400 (M.S.P.), the Wilson Foundation, the Shalom Foundation, the Skirball Foundation, and NHLBI 1RO1-HL84142 (K.R.L.).

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