Published in final edited form as:

Am J Physiol Heart Circ Physiol. 2007 October ; 293(4): H2129–H2139. doi:10.1152/ajpheart.00677.2007.

Skeletal myoblasts transplanted in the ischemic myocardium enhance in situ oxygenation and recovery of contractile function

Mahmood Khan^{1,*}, Vijay Kumar Kutala^{1,*}, Deepti S. Vikram¹, Sheik Wisel², Simi M. Chacko¹, M. Lakshmi Kuppusamy¹, Iyyapu K. Mohan¹, Jay L. Zweier¹, Pawel Kwiatkowski², Periannan Kuppusamy¹

¹Center for Biomedical Electron Paramagnetic Resonance Spectroscopy and Imaging, Davis Heart and Lung Research Institute, Division of Cardiovascular Medicine, Department of Internal Medicine

²Division of Cardiothoracic Surgery, Department of Surgery; The Ohio State University, Columbus, Ohio

Abstract

It is unclear whether oxygen plays a role in stem cell therapy. Hence, the determination of local oxygenation (Po_2) in the infarct heart and at the site of transplantation may be critical to study the efficacy of cell therapy. To demonstrate this, we have developed an oxygen-sensing paramagnetic spin probes (OxySpin) to monitor oxygenation in the region of cell transplantation using electron paramagnetic resonance (EPR) spectroscopy. Skeletal myoblast (SM) cells isolated from thigh muscle biopsies of mice were labeled with OxySpin by coculturing the cells with submicron-sized (270 ± 120 nm) particulates of the probe. Myocardial infarction was created by left coronary artery ligation in mice. Immediately after ligation, labeled SM cells were transplanted in the ischemic region of the heart. The engraftment of the transplanted cells and in situ Po_2 in the heart were monitored weekly for 4 wk. EPR measurements revealed the retention of cells in the infarcted tissue. The myocardial Po_2 at the site of SM cell therapy was significantly higher compared with the untreated group throughout the 4-wk period. Histological studies revealed differentiation and engraftment of SM cells into myotubes and increased incidence of neovascularization in the infarct region. The infarct size in the treated group was significantly decreased, whereas echocardiography showed an overall improvement in cardiac function when compared with untreated hearts. To our knowledge, this is the first report detailing changes in in situ oxygenation in cell therapy. The increased myocardial Po_2 positively correlated with neoangiogenesis and cardiac function.

Keywords

ischemic heart; myocardial infarction; electron paramagnetic resonance oximetry; OxySpin

Address for reprint requests and other correspondence: P. Kuppusamy, Davis Heart and Lung Research Inst., The Ohio State Univ., 420 W. 12th Ave., Rm. 114, Columbus, OH 43210 (kuppusamy.1@osu.edu).

*M. Khan and V. K. Kutala contributed equally to this work. V. K. Kutala was on sabbatical from Nizam's Institute of Medical Sciences, Hyderabad, India.

ACUTE MYOCARDIAL INFARCTION (AMI) leads to impaired cardiac function, which is a major cause of morbidity and mortality (9). Although a variety of surgical interventions are available to rescue the failing heart, cellular cardiomyoplasty, wherein the lost cells are replaced by transplantation of stem cells in the affected region, is being perceived as a potential alternative. The most extensively studied cell types for cardiac tissue regeneration (repair) are bone marrow-derived stem cells (BMCs), endothelial progenitor cells, and skeletal myoblast (SM) cells (14, 21, 24). A number of studies have shown the beneficial effects of stem cell transplantation on cardiac function after AMI (21, 24, 40). Adult BMCs, injected into the peri-infarcted myocardium of left anterior descending coronary artery (LAD)-ligated mice, have shown significantly improved cardiac function (24). Grafting of autologous SM cells into ischemic hearts and transplantation of SM cells in animal models of AMI have improved cardiac performance and graft survival (29, 32, 37, 44). However, clinical studies of stem cell therapy in cardiovascular patients have yielded mixed results. Patients with AMI, treated with BMCs or SMs, have exhibited a significant increase in left ventricular (LV) contractile function (3, 7, 22, 26, 32). On the other hand, other studies have shown no improvement using mononuclear BMCs (17).

The grafting of myoblasts, fetal cardiomyocytes, or embryonic or bone marrow-derived stem cells onto myocardial scar tissue has resulted in marginal improvements in cardiac function and in the limitation of abnormal cardiac remodeling (1, 6, 10, 13, 24, 26, 30, 38). The limited survival of the transplanted cells in the infarcted myocardium is thought to be the reason for such modest improvements (18). The hypovascular nature of the infarcted tissue may severely compromise the availability of oxygen, nutrients, and growth factors essential for the survival and differentiation of the transplanted cells. The local hypoxic environment in the infarcted myocardium may be the main impediment to the survival of the transplanted cells. However, it is not clear whether the oxygen concentration in the ischemic myocardium (infarcted area) is affected by strategies that stimulate angiogenesis and/or by cell transplantation. It is also unknown whether there is a relationship between oxygen concentration and transplanted cell survival. Oxygen tension plays an important role in the growth of stem cells in culture and significantly influences their expansion and differentiation (8, 31, 39). In response to acute hypoxia, cardiomyocytes have been shown to exhibit adaptations that may facilitate cell survival and develop tolerance to subsequent acute severe hypoxia (33). Hence, the determination of in situ oxygenation at the transplant site within the ischemic heart tissue is vital for the understanding of the effects of cell therapy.

Although several existing methods are used to measure oxygen concentration, a suitable technique for noninvasive and repeated measurements of oxygen in the same tissue or cells on a temporal scale is not available. Electron paramagnetic resonance (EPR) spectroscopy, a technique commonly used for direct detection of free radicals and paramagnetic species, has recently been adapted to make reliable and accurate determination of the concentration of molecular oxygen (oximetry) (35). EPR oximetry refers to the measurement of the partial pressure of oxygen (P_{O_2}) by EPR spectroscopy. The principle of EPR oximetry is based on the paramagnetic characteristics of molecular oxygen, which in its ground state has two unpaired electrons, and undergoes spin exchange interaction with the paramagnetic EPR spin probe. This process is sensitive to oxygen content, with the relaxation rate of the spin

probe increasing as a function of oxygen content (concentration/pressure). This increased spin-spin relaxation rate results in increased line broadening that is directly proportional to the oxygen content. The EPR technique requires the incorporation of an oxygen-sensing paramagnetic spin probe into the tissue of interest. The particulate probes measure Po_2 in the tissue milieu. Lithium octa-*n*-butoxy-substituted naphthalocyanine radical (LiNc-BuO) is a particulate oximetry spin probe that we have recently synthesized and validated for in vivo oximetry (2, 15, 27, 42, 43). The LiNc-BuO crystals are composed of stacks of neutral radicals of lithiated naphthalocyanine macrocycles (28). The EPR spectra of these particulates are characterized by a single and very narrow absorption peak due to the strong exchange coupling between the unpaired electrons within the molecular stack. The probe, in the form of submicron-sized (270 ± 120 nm) crystals (hereafter referred to as OxySpin), can be internalized in cells without compromising cell integrity and function (15, 27). A unique advantage of this probe is that it is retained in cells/tissues for substantially long periods, thus enabling continuous monitoring of Po_2 for several months and possibly longer (27). This prompted us to further develop this unique technology for stem cell therapy, where the long-term monitoring of local tissue Po_2 at the transplanted site would be highly useful for investigating the efficacy of cell therapy.

Accordingly, the goal of this work was to use the noninvasive EPR technology to monitor in situ Po_2 for weeks after cell transplantation in a mouse model of AMI. In this study, we used SM cells because of their innate ability to propagate in large numbers, their resistance to hypoxia, and the ease of isolation from skeletal muscle biopsies (4, 11, 12, 23, 29). The EPR results clearly established the feasibility of measuring in situ Po_2 from the engraftment site in vivo. On the whole, the results showed the retention of SM cells and the differentiation of the cells into myotubes, neovascularization in the infarct region, and a significant increase in Po_2 at the site of engraftment with an overall improvement in cardiac function.

MATERIALS AND METHODS

Isolation and labeling of SMs with OxySpin

SMs were isolated from the hindlimb skeletal muscle biopsies of mice and cultured as described previously (42). The cells were coincubated with a suspension of sonicated OxySpin crystals for 48 h (15, 42). We have recently reported that myoblasts labeled with or coincubated in the presence of OxySpin probes did not show any significant change in viability, metabolism, or proliferation rate (42). A stock suspension of $\sim 6 \times 10^6$ cells in 1 ml of sterile PBS containing glucose (1 mg/ml) was used for implantation in hearts.

Induction of myocardial ischemia and cell transplantation

Male C57BL mice (25–30 g) were used. The myocardial infarction (MI) in mice was created by permanently occluding the left anterior descending coronary artery (LAD, also known as the left coronary artery in mouse) as described (41, 42). An oblique 8-mm incision was made 2 mm away from the left sternal border toward the left armpit. The chest cavity was opened with scissors by a small incision (5 mm in length) at the level of the third or fourth intercostal space 2 to 3 mm from the left sternal border. The LAD was visualized as a pulsating bright red spike running through the midst of the heart wall from underneath the

left atrium toward the apex. The LAD was ligated 1 to 2 mm below the tip of the left auricle using a tapered needle and an 8–0 polypropylene ligature passed underneath the LAD, and a double knot was made to occlude the LAD. Occlusion was confirmed by the dramatic change in color (pale) of the anterior wall of the LV. The chest cavity was closed by bringing together the third and fourth ribs with one 6–0 polypropylene silk suture. The layers of muscle and skin were closed with a 5–0 polypropylene suture. After LAD ligation, an ST elevation on EKG and a color change in the LV myocardium were recognized in all mice. All the procedures were performed with the approval of the Institutional Animal Care and Use Committee of the Ohio State University and conformed to the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health (NIH Publication No. 86–23, Revised 1996).

A single injection of the labeled SMs (1×10^5 cells in 15 μ l culture medium) was performed into the midventricular region of hearts immediately after LAD ligation (MI + SM group). The non-MI control group of animals received OxySpin only (in 15 μ l culture medium) without LAD ligation. The MI group received OxySpin only immediately after LAD ligation. The chest was closed after implantation of the cells. Oxygen measurements were performed immediately and then every week for 4 wk using in vivo EPR oximetry.

EPR oximetry of myocardial Po_2

Measurements of myocardial oxygenation were performed noninvasively using an L-band in vivo EPR spectrometer (L-band, Magnettech) equipped with automatic coupling and tuning controls for measurements in beating hearts. Mice, under anesthesia (2% isoflurane), were placed in a right lateral position with their chest close to the loop of the surface coil resonator. EPR spectra were acquired as single 30-s scans. The instrument settings were as follows: incident microwave power, 4 mW; modulation amplitude, 180 mG, modulation frequency, 100 kHz; and receiver time constant, 0.2 s. The peak-to-peak width of the EPR spectrum was used to calculate Po_2 using a standard calibration curve (27).

EPR and MRI

EPR images of OxySpin in the ex vivo hearts were obtained using an L-band (1.2 GHz) EPR imager. The imaging of the heart morphology was done by postlabeling the excised formalin-fixed hearts with a triarylmethyl radical (TAM, Ox063, 1.0 mM) in PBS for 12 h. The rationale behind this was that the TAM label would enter the formalin-fixed heart and, therefore, would provide an anatomical mapping of the entire heart. A modulation-weighted imaging procedure was used to acquire and coregister the images of OxySpin and TAM in the same heart. The instrument settings were as follows: incident microwave power, 4 mW; modulation amplitude, 790 mG; sweep width, 16 G; scan time of 7.86 s/projection, 32×32 projections; and magnetic field, gradient 8 G/cm.

The OxySpin-induced paramagnetic contrast in proton MRI was used to visualize the probes in high-resolution images of the heart using a 500 MHz (11.7 T) Bruker MRI system. The images were acquired using a FLASH sequence with the following parameters: field of view, 10×10 mm; slice thickness, 0.4 mm; repetition time (TR), 700 ms; echo time (TE), 12 ms;

pixels 128×128 ; number of averages [number of excitations (NEX)] 32; and flip angle, 5° . The same hearts were used for EPR and MRI imaging.

Echocardiography

Mice were kept under isoflurane (2%) anesthesia. Two-dimensional images were obtained from mice orientated on a heating pad in a left lateral decubitus or supine position. LV parameters were obtained from M-mode interrogation in a long-axis view: interventricular septum thickness, LV posterior wall thickness, LV internal diastolic diameter (LVIDd), and LV internal systolic diameter (LVIDs). LV percent fractional shortening (LV%FS) and LV ejection fraction (LVEF) were calculated as follows: $\text{LV\%FS} = (\text{LVIDd} - \text{LVIDs})/\text{LVIDd} \times 100$; and $\text{LVEF} = [(\text{LVIDd})^3 - (\text{LVIDs})^3]/(\text{LVIDd})^3 \times 100$. All echocardiographic measurements were averaged from at least three separate cardiac cycles.

Determination of cardiac function

Hemodynamic measurements were performed in animals at 4 wk after the induction of MI. Mice were kept under isoflurane (2%) anesthesia. A Millar catheter (SPR1000) was advanced through the right carotid artery into the LV. The LV systolic pressure (LVSP), LV end-diastolic pressure (LVEDP), and maximum rate of increase ($+dP/dt_{\max}$) and decrease ($-dP/dt_{\max}$) of LVSP were recorded and analyzed using a PowerLab data acquisition system (model ML866; ADInstruments, Colorado Springs, CO).

Measurement of infarct size and fibrosis

At the end of experimental period, animals were euthanized with an overdose of pentobarbital sodium. The hearts were removed immediately and attached to a Langendorff apparatus. Triphenyltetrazolium chloride (1.5%) solution was injected down the side arm of the aortic cannula and infused into the coronary circulation. Once the hearts were stained dark red, they were removed, weighed, and frozen. The following day, the hearts were defrosted and sliced into 1-mm sections parallel to the atrioventricular groove and then fixed in 10% buffered formalin for overnight. The images of slices were scanned with a flatbed scanner, and the area of infarction and total ventricular zone were planimetered using an image analysis software (NIH Image). Infarct size (% of LV) was calculated as $(\text{infarct area}/\text{total LV area}) \times 100$.

For measurement of fibrosis area, after measurement of the infarct size, heart tissues were fixed with 10% buffered formalin and embedded in paraffin. Paraffin sections were stained with Masson's trichrome stain. Images of the LV area of each slide were prepared by Nikon Model C-PS (objective $\times 20$) with Spot Insight camera (Diagnostic). Fibrosis and total LV area of each image were measured using the Metamorph software.

Histological and immunohistochemical analysis

Hearts were fixed in formalin and embedded in paraffin. Sections ($6 \mu\text{m}$) were obtained and used for hematoxylin and eosin staining. For immunofluorescence staining, the formalin-fixed and paraffin-embedded tissue sections (8 μm thickness) were serially rehydrated in 100%, 95%, and 80% ethanol after deparaffinization with xylene. Slides were kept in steam for 30 min and then washed in PBS (pH 7.4) three times for 5 min each. Tissue sections

Author Manuscript

Author Manuscript

Author Manuscript

were incubated with 2% goat serum and 5% bovine serum albumin in PBS to reduce nonspecific binding. The sections were then incubated for 4 h with an anti-mouse anti-von Willebrand factor VIII monoclonal antibody (vWF, LabVision, Fremont, CA) for the determination of capillary density, MY-32 (1:300, Sigma) for staining SMs, anti- α -smooth muscle actin (α -SMA) mouse antibody (LabVision) for blood vessels, or anti-VEGF (final concentration 15 μ g/ml, Sigma) for VEGF expression. The sections were then incubated with secondary antibodies (1:1,000 dilution) conjugated to Texas red (α -SMA), peroxidase (MY-32, vWF VIII), or FITC (VEGF). Nuclei were counterstained with 4',6-diamino-2-phenylindole. The tissue slides were visualized using a Nikon fluorescence microscope. Separate sections were also stained without primary antibodies to examine nonspecific binding. The blood vessels positive for vWF VIII, and α -SMAs were counted in both infarct and peri-infarct regions. At least 32 high-power microscopic fields ($\times 400$) in both infarct and peri-infarct regions were randomly selected and counted in each treatment group of animals ($n = 4$ animals/group) using Metamorph software. Immunohistochemical analysis was performed using diaminobenzidine substrate kit (Vector, Burlingame, CA). Blood vessel density was expressed as the number of vessels per surface area (0.155 mm²). The number of capillaries was counted in the infarcted region and border zone. Capillary density was expressed as the average number of capillaries in five random high-power fields.

Measurement of wall thickness and LV internal diameter

After the hemodynamic measurements, animals were euthanized and the hearts were removed and washed two to three times in cold PBS. The excised hearts were then cut into three transverse slices. Each slice was fixed in 4% paraformaldehyde and embedded in paraffin. The middle transverse section was stained with hematoxylin and eosin and Masson's trichrome for both infarct-size and wall-thickness measurements. 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. The mean wall thickness of infarcted myocardium was measured from three equidistant points. Quantitative assessment of each parameter was performed using Metamorph software.

Statistical analysis

All data are represented as means $\pm$ SD. Statistical evaluations were performed using SAS software (SAS Institute, Cary, NC). Tukey's multiple-comparison procedure was used to adjust P value for post hoc pairwise comparisons. In all cases, $P < 0.05$ was deemed statistically significant.

RESULTS

Visualization of labeled SM cells in the intact heart by EPR and MRI

EPR and MRI were used to visualize the distribution of OxySpin in the intact heart, ex vivo. The excised hearts were postlabeled with a water-soluble paramagnetic label for visualization using EPR imaging. The OxySpin-induced paramagnetic contrast in proton MRI was used to visualize the presence of the probes in the high-resolution MRI images of the heart. Figure 1 shows the EPR and MR images of hearts at 4 wk after transplantation of

cells labeled with OxySpin. The images established the presence and localization of OxySpin at the therapeutic site after 4 wk of implantation in the infarcted heart of mice.

In vivo detection of transplanted SM cells in the heart

The OxySpin-labeled SM cells transplanted in the infarcted heart were monitored noninvasively, using in vivo EPR spectroscopy. The EPR spectrum of the OxySpin probes is characterized by a single narrow absorption peak. The OxySpin suspended in PBS showed a line width of 1.98 G in room air (Fig. 2A) and 0.37 G in 0% oxygen (Fig. 2B), with a linear dependence of peak-to-peak line width on P_{O_2} (data not shown). Figure 2, C and D, shows the EPR spectra obtained from a mouse heart, in vivo, at 1 day and 2 wk, respectively, after transplantation of OxySpin-labeled SM cells in the infarcted region. The results suggest that the probe is retained in the heart, enabling precise monitoring of oxygen concentration in the heart tissue.

SM treatment enhanced myocardial P_{O_2} at the therapeutic site of infarcted hearts

Noninvasive and repeated measurements of myocardial tissue P_{O_2} in the infarcted region of hearts were performed for 4 wk using in vivo EPR oximetry. Figure 3 shows the time course of myocardial P_{O_2} measured from infarcted hearts (MI) and infarcted hearts treated with SM cells (MI + SM) implanted in the ischemic region. The mean baseline P_{O_2} in the noninfarct control hearts was 15.0 ± 1.4 mmHg ($n = 7$) during the 1–4 wk period (data not shown). A marked decrease of myocardial P_{O_2} was observed in the MI group (Fig. 3A). There was no significant difference between the baseline (*day 0*) P_{O_2} values of MI and MI + SM groups. The P_{O_2} remained low throughout the 4 wk after induction of MI (Fig. 3B). In the case of infarct hearts treated with SM cells (MI + SM group), the myocardial P_{O_2} was significantly higher at 1, 2, 3, and 4 wk after SM transplantation compared with the MI group ($P < 0.05$).

SM treatment improves functional recovery of infarcted hearts

Four weeks after LAD ligation, hemodynamic studies were performed by echocardiography in the three groups (Fig. 4). LV ejection fraction (LVEF) was significantly decreased in the MI group compared with control ($P < 0.01$). The LVEF was significantly improved in the treated group (SM + MI) compared with the MI group ($P < 0.05$). Similarly, LV fractional shortening (FS) was decreased in the MI group compared with control ($P < 0.01$), whereas the decrease was significantly attenuated in the SM-treated group ($P < 0.05$ vs. MI group). LV internal dimensions at the end diastole (LVIDd), a marker of LV remodeling, was significantly increased ($P < 0.05$) in the MI group compared with the MI + SM group (0.48 ± 0.05 vs. 0.39 ± 0.01 cm; $P \pm 0.05$). Similarly LV internal dimensions at end systole (LVIDs) was significantly higher in the MI group compared with the MI + SM group of hearts (0.35 ± 0.04 vs. 0.26 ± 0.02 cm; $P < 0.05$). The SM-treated group also showed a significantly higher LVSP and a significantly lower LVEDP compared with those in the MI group (Fig. 5). The $dP/dt_{\max}$ was significantly higher, and $-dP/dt_{\max}$ was lower, in the treated group. The transplantation of SM cells significantly improved ventricular contractility reflected by the increase in dP/dt and LVSP. There was no significant difference in heart rates between the three groups (Fig. 5).

Histology and immunohistochemical studies

Representative histology and immunohistochemical sections of heart tissues at 1, 2, or 4 wk after cell transplantation are shown in Fig. 6. Figure 6, A and B, shows the hematoxylin and eosin and MY-32 staining of noninfarcted control hearts. Tissue sections show positive MY-32 (skeletal muscle cell-specific heavy chain) staining in the infarcted myocardium at 1 wk (Fig. 6, C and D), 2 wk (Fig. 6, E and F), and 4 wk (Fig. 6G) after SM transplantation. Positive MY-32 staining was found in the infarcted myocardium as early as 1 wk after SM transplantation (Fig. 6D). MY-32 expression was not detected earlier than 1 wk after implantation (data not shown). Examination of cell grafts at higher magnification indicated that, at 4 wk after transplantation, the cells developed an elongated morphology, characteristic of fused polynucleated myotubes (Fig. 6G). Hematoxylin and eosin and Masson's trichrome staining showed the presence of inflammatory cells and fibrous tissue 4 wk after SM treatment in the transplanted areas (data not shown).

SM treatment induces neovascularization in the infarct heart

To investigate whether the increased myocardial P_{O_2} following SM transplantation was due to increased neoangiogenesis, we assessed VEGF expression in the border region and used anti-factor VIII and anti- α -SMA-antibodies to determine capillary density and vessel formation in the center and border region of the infarct zone (Fig. 7). Both capillaries and vessels were found in the infarct and border area in SM transplanted and MI groups. The SM-transplanted group showed significantly enhanced angiogenesis with a large number of capillaries found in the infarct and border zones compared with untreated hearts. There was a significant increase in VEGF fluorescence intensity in the MI + SM group compared with MI group (4.8 ± 0.58 vs. 1.5 ± 0.22 ; $P < 0.05$). Capillary density of the transplanted group in the infarct and border zones was 4.5 ± 1.3 and 9.7 ± 1.7 and in the MI group was 2.0 ± 0.8 and 6.7 ± 0.9 /high power field, respectively.

SM treatment decreases myocardial infarct size and LV remodelling

The myocardial infarct size was significantly smaller in the SM-treated group compared with the MI group ($P < 0.05$) (Fig. 8). In addition, the SM-treated group showed a significantly smaller LV internal diameter ($P < 0.05$) and a greater LV wall thickness ($P < 0.05$) compared with those in the MI group (Fig. 8).

DISCUSSION

The present study represents two significant advances in our ability to monitor stem cell therapy. The first is the establishment of an oxygen-sensing probe-based in vivo EPR technology for accurate measurements of myocardial oxygenation (P_{O_2}) as it relates to stem cell therapy. The measurements are performed noninvasively and repeatedly for weeks following implantation. The second is the finding that SMs increase myocardial oxygenation at the site of implantation in the infarct heart, which positively correlated with cell engraftment, promotion of neoangiogenesis, and improved cardiac function. To our knowledge, this is the first report demonstrating the changes in in situ oxygenation in the infarcted heart after cell transplantation. The EPR technique is particularly advantageous in offering high-sensitivity detection of the spin probes and determining the absolute value of

the local oxygen content. The monitoring of local tissue Po_2 at the site of engraftment is important to determine the crucial role (for example, causal vs. consequential) of oxygen in stem-cell survival and tissue repair.

The results of the present study clearly established that the ischemic region was significantly hypoxic for 4 wk after the induction of ischemia. Furthermore, there was no significant change in tissue Po_2 during the development of LV remodeling. Transplantation of SM cells in the ischemic region showed a significant increase in Po_2 compared with that in untreated tissue. Therefore, it is inferred that the transplanted SM may be responsible for the augmented myocardial Po_2 in the infarcted heart. Overall we observed an increase of ~ 2 mmHg, which corresponds to more than $2.5 \mu\text{M}$ of higher oxygen concentration at the therapeutic site. Thus the magnitude of increase in oxygenation, albeit being small, may still be sufficient for the survival of cells in the infarcted region. However, the exact mechanism of increased myocardial Po_2 after SM cell transplantation is not known at present. It is possible that growth factors released by the transplanted cells may enhance angiogenesis within the infarcted and noninfarcted regions (16). Transplanted SM cells are known to secrete VEGF and induce angiogenesis (34). Thus the VEGF expression, observed in the infarct region of SM-treated hearts, could explain the higher capillary density and vessel formation. It is likely that the newly formed vessels might improve tissue perfusion in and around the ischemic zone, resulting in increased blood flow and myocardial Po_2 . Several studies have demonstrated that expression of certain growth factors, including VEGF, by the transplanted cells is a possible mechanism for functional improvement in the heart (19, 25, 34). SMs expressing VEGF have been shown to induce capillary formation and reduce cardiac injury (5).

The differentiation and engraftment of the transplanted SMs was confirmed by histological analysis. At 1 wk, differentiated SM cells were identified with the expression of MY-32 within the infarcted region. A similar observation has been reported in rats (36), sheep (18), and humans (26) by other investigators. In addition, the histological analysis showed the presence of OxySpin in the engrafted site. There was an early inflammatory response observed at the site of implantation at 2 wk, and the presence of fibrous tissue was observed after 4 wk in all groups (data not shown).

SM transplantation improved cardiac function at 4 wk after MI, as indicated by a significant increase in LVSP and decrease in LVEDP. The treatment also significantly improved the ventricular contractility as reflected by the increase in $\text{dP/dt}_{\text{max}}$ and a decrease in $-\text{dP/dt}_{\text{max}}$. This improvement in cardiac function may be attributed to a decrease of LV remodeling due to SM transplantation. Our results are in agreement with several studies that reported the beneficial effects of SM cells in the treatment of LV dysfunction in animals and humans (20, 22, 25, 29, 32, 37). The results of the present study also indicated that SM transplantation significantly reduced the infarct size and attenuated wall thinning, which could be attributed to SM-induced neovascularization. Taken together, the increase in tissue perfusion due to SM-induced neovascularization, the decrease in infarct size, and an increased myocardial Po_2 may be the factors responsible for the benefits of SM cells against postinfarction remodeling. Our results clearly indicated that changes in myocardial oxygenation could be used as an indicator of the outcome of cell therapy.

The present method also acknowledges certain limitations in our ability to quantify cell count and cell migration. The first limitation is that the fate of the intracellular OxySpin following cell death is not known. The second limitation is the inability to perform EPR imaging of the location and distribution of labeled SM cells in the beating heart, in vivo, due to heart motion. A cardiac-gated technique to overcome the motion-induced limitation is currently under development. Finally, further advances in the EPR instrumentation is required to perform the measurements in large animals, including humans.

In summary, we were able to demonstrate, for the first time, the feasibility of in vivo monitoring of myocardial Po_2 at the site of stem-cell therapy. We observed an increase in the myocardial Po_2 in hearts treated with SMs that positively correlated with increased neoangiogenesis and improvement in cardiac function.

Acknowledgments

GRANTS

This work was supported by National Institutes of Health Grant R01-EB-006163. The development of the probe (LiNc-BuO) for EPR oximetry was supported by National Institutes of Health Grant R01-EB-004031.

REFERENCES

1. Agbulut O, Vandervelde S, Al Attar N, Larghero J, Ghostine S, Leobon B, Robidel E, Borsani P, Le Lor'h M, Bissery A, Chomienne C, Bruneval P, Marolleau JP, Vilquin JT, Hagege A, Samuel JL, Menasche P. Comparison of human skeletal myoblasts and bone marrow-derived CD133⁺ progenitors for the repair of infarcted myocardium. *J Am Coll Cardiol* 44: 458–463, 2004. [PubMed: 15261948]
2. Angelos MG, Kutala VK, Torres CA, He G, Stoner JD, Mohammad M, Kuppusamy P. Hypoxic reperfusion of the ischemic heart and oxygen radical generation. *Am J Physiol Heart Circ Physiol* 290: H341–H347, 2006. [PubMed: 16126819]
3. Assmus B, Honold J, Schachinger V, Britten MB, Fischer-Rasokat U, Lehmann R, Teupe C, Pistorius K, Martin H, Abolmaali ND, Tonn T, Dimmeler S, Zeiher AM. Transcoronary transplantation of progenitor cells after myocardial infarction. *N Engl J Med* 355: 1222–1232, 2006. [PubMed: 16990385]
4. Atkins BZ, Huelman MT, Meuchel JM, Cottman MJ, Hutcheson KA, Taylor DA. Myogenic cell transplantation improves in vivo regional performance in infarcted rabbit myocardium. *J Heart Lung Transplant* 18: 1173–1180, 1999. [PubMed: 10612375]
5. Becker C, Lacchini S, Muotri AR, da Silva GJ, Castelli JB, Vassallo PF, Menck CF, Krieger JE. Skeletal muscle cells expressing VEGF induce capillary formation and reduce cardiac injury in rats. *Int J Cardiol* 113: 348–354, 2006. [PubMed: 16675047]
6. Chiu RC, Zibaitis A, Kao RL. Cellular cardiomyoplasty: myocardial regeneration with satellite cell implantation. *Ann Thorac Surg* 60: 12–18, 1995. [PubMed: 7598572]
7. Drexler H, Meyer GP, Wollert KC. Bone-marrow-derived cell transfer after ST-elevation myocardial infarction: lessons from the BOOST trial. *Nat Clin Pract Cardiovasc Med* 3, Suppl 1: S65–S68, 2006. [PubMed: 16501634]
8. Ezashi T, Das P, Roberts RM. Low O_2 tensions and the prevention of differentiation of hES cells. *Proc Natl Acad Sci USA* 102: 4783–4788, 2005. [PubMed: 15772165]
9. Fuster V, Badimon L, Badimon JJ, Chesebro JH. The pathogenesis of coronary artery disease and the acute coronary syndromes (1). *N Engl J Med* 326: 242–250, 1992. [PubMed: 1727977]
10. Ghostine S, Carrion C, Souza LC, Richard P, Bruneval P, Vilquin JT, Pouzet B, Schwartz K, Menasche P, Hagege AA. Long-term efficacy of myoblast transplantation on regional structure and function after myocardial infarction. *Circulation* 106: I131–I136, 2002. [PubMed: 12354722]

11. Hutcheson KA, Atkins BZ, Hueman MT, Hopkins MB, Glower DD, Taylor DA. Comparison of benefits on myocardial performance of cellular cardiomyoplasty with skeletal myoblasts and fibroblasts. *Cell Transplant* 9: 359–368, 2000. [PubMed: 10972335]
12. Jain M, DerSimonian H, Brenner DA, Ngoy S, Teller P, Edge AS, Zawadzka A, Wetzel K, Sawyer DB, Colucci WS, Apstein CS, Liao R. Cell therapy attenuates deleterious ventricular remodeling and improves cardiac performance after myocardial infarction. *Circulation* 103: 1920–1927, 2001. [PubMed: 11294813]
13. Kamihata H, Matsubara H, Nishiue T, Fujiyama S, Tsutsumi Y, Ozono R, Masaki H, Mori Y, Iba O, Tateishi E, Kosaki A, Shintani SM,T, Imaizumi T, Iwasaka T Implantation of bone marrow cells into ischemic myocardium enhances collateral perfusion and regional function via side supply of angioblasts, angiogenic ligands, and cytokines. *Circulation* 104: 1046–1052, 2001. [PubMed: 11524400]
14. Kocher AA, Schuster MD, Szabolcs MJ, Takuma S, Burkhoff D, Wang J, Homma S, Edwards NM, Itescu S. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. *Nat Med* 7: 430–436, 2001. [PubMed: 11283669]
15. Kutala VK, Parinandi NL, Pandian RP, Kuppusamy P. Simultaneous measurement of oxygenation in intracellular and extracellular compartments of lung microvascular endothelial cells. *Antioxid Redox Signal* 6: 597–603, 2004. [PubMed: 15130286]
16. Leor J, Prentice H, Sartorelli V, Quinones MJ, Patterson M, Kedes LK, Kloner RA. Gene transfer and cell transplant: an experimental approach to repair a ‘broken heart.’ *Cardiovasc Res* 35: 431–441, 1997. [PubMed: 9415287]
17. Lunde K, Solheim S, Aakhus S, Arnesen H, Abdelnoor M, Egeland T, Endresen K, Ilebekk A, Mangschau A, Fjeld JG, Smith HJ, Taraldsrud E, Grogard HK, Bjornerheim R, Brekke M, Muller C, Hopp E, Ragnarsson A, Brinchmann JE, Forfang K. Intracoronary injection of mononuclear bone marrow cells in acute myocardial infarction. *N Engl J Med* 355: 1199–1209, 2006. [PubMed: 16990383]
18. McConnell PI, del Rio CL, Jacoby DB, Pavlicova M, Kwiatkowski P, Zawadzka A, Dinsmore JH, Astra L, Wisel S, Michler RE. Correlation of autologous skeletal myoblast survival with changes in left ventricular remodeling in dilated ischemic heart failure. *J Thorac Cardiovasc Surg* 130: 1001, 2005. [PubMed: 16214511]
19. Menasche P Myoblast transfer in heart failure. *Surg Clin North Am* 84: 125–139, 2004. [PubMed: 15053186]
20. Menasche P, Hagege AA, Scorsin M, Pouzet B, Desnos M, Duboc D, Schwartz K, Vilquin JT, Marolleau JP. Myoblast transplantation for heart failure. *Lancet* 357: 279–280, 2001. [PubMed: 11214133]
21. Min JY, Sullivan MF, Yang Y, Zhang JP, Converso KL, Morgan JP, Xiao YF. Significant improvement of heart function by cotransplantation of human mesenchymal stem cells and fetal cardiomyocytes in postinfarcted pigs. *Ann Thorac Surg* 74: 1568–1575, 2002. [PubMed: 12440610]
22. Murry CE, Wiseman RW, Schwartz SM, Hauschka SD. Skeletal myoblast transplantation for repair of myocardial necrosis. *J Clin Invest* 98: 2512–2523, 1996. [PubMed: 8958214]
23. Nian M, Lee P, Khaper N, Liu P. Inflammatory cytokines and postmyocardial infarction remodeling. *Circ Res* 94: 1543–1553, 2004. [PubMed: 15217919]
24. Orlic D, Kajstura J, Chimenti S, Bodine DM, Leri A, Anversa P. Transplanted adult bone marrow cells repair myocardial infarcts in mice. *Ann NY Acad Sci* 938: 221–229, 2001. [PubMed: 11458511]
25. Oshima H, Payne TR, Urish KL, Sakai T, Ling Y, Gharaibeh B, Tobita K, Keller BB, Cummins JH, Huard J. Differential myocardial infarct repair with muscle stem cells compared to myoblasts. *Mol Ther* 12: 1130–1141, 2005. [PubMed: 16125468]
26. Pagani FD, DerSimonian H, Zawadzka A, Wetzel K, Edge AS, Jacoby DB, Dinsmore JH, Wright S, Aretz TH, Eisen HJ, Aaronson KD. Autologous skeletal myoblasts transplanted to ischemia-damaged myocardium in humans. Histological analysis of cell survival and differentiation. *J Am Coll Cardiol* 41: 879–888, 2003. [PubMed: 12628737]

27. Pandian RP, Parinandi NL, Ilangoan G, Zweier JL, Kuppusamy P. Novel particulate spin probe for targeted determination of oxygen in cells and tissues. *Free Radic Biol Med* 35: 1138–1148, 2003. [PubMed: 14572616]
28. Pandian RP, Kim Y, Woodward P, Zweier JL, Manoharan PT, Kuppusamy P. Open molecular framework of paramagnetic lithium octabutoxy-naphthalocyanine: implications for the detection of oxygen and nitric oxide using EPR spectroscopy. *J Mater Chem* 16: 3609–3618, 2006.
29. Pouzet B, Hagege AA, Vilquin JT, Desnos M, Duboc D, Marolleau JP, Menashe P. Transplantation of autologous skeletal myoblasts in ischemic cardiac insufficiency. [In French.] *J Soc Biol* 195: 47–49, 2001. [PubMed: 11530499]
30. Retuerto MA, Schalch P, Patejunas G, Carbray J, Liu N, Esser K, Crystal RG, Rosengart TK. Angiogenic pretreatment improves the efficacy of cellular cardiomyoplasty performed with fetal cardiomyocyte implantation. *J Thorac Cardiovasc Surg* 127: 1041–1049, 2004. [PubMed: 15052201]
31. Salim A, Giaccia AJ, Longaker MT. Stem cell differentiation. *Nat Biotechnol* 22: 804–805; author reply 805–806, 2004. [PubMed: 15229534]
32. Scorsin M, Hagege A, Vilquin JT, Fiszman M, Marotte F, Samuel JL, Rappaport L, Schwartz K, Menashe P. Comparison of the effects of fetal cardiomyocyte and skeletal myoblast transplantation on postinfarction left ventricular function. *J Thorac Cardiovasc Surg* 119: 1169–1175, 2000. [PubMed: 10838534]
33. Silverman HS, Wei S, Haigney MC, Ocampo CJ, Stern MD. Myocyte adaptation to chronic hypoxia and development of tolerance to subsequent acute severe hypoxia. *Circ Res* 80: 699–707, 1997. [PubMed: 9130451]
34. Suzuki K, Murtuza B, Smolenski RT, Sammut IA, Suzuki N, Kaneda Y, Yacoub MH. Cell transplantation for the treatment of acute myocardial infarction using vascular endothelial growth factor-expressing skeletal myoblasts. *Circulation* 104: I207–I212, 2001. [PubMed: 11568057]
35. Swartz HM, Clarkson RB. The measurement of oxygen in vivo using EPR techniques. *Phys Med Biol* 43: 1957–1975, 1998. [PubMed: 9703059]
36. Tambara K, Sakakibara Y, Sakaguchi G, Lu F, Premaratne GU, Lin X, Nishimura K, Komeda M. Transplanted skeletal myoblasts can fully replace the infarcted myocardium when they survive in the host in large numbers. *Circulation* 108, Suppl 1: II259–II263, 2003. [PubMed: 12970243]
37. Taylor DA, Atkins BZ, Hungspreugs P, Jones TR, Reedy MC, Hutcheson KA, Glower DD, Kraus WE. Regenerating functional myocardium: improved performance after skeletal myoblast transplantation. *Nat Med* 4: 929–933, 1998. [PubMed: 9701245]
38. Tse HF, Kwong YL, Chan JK, Lo G, Ho CL, Lau CP. Angiogenesis in ischaemic myocardium by intramyocardial autologous bone marrow mononuclear cell implantation. *Lancet* 361: 47–49, 2003. [PubMed: 12517468]
39. Wang DW, Fermor B, Gimble JM, Awad HA, Guilak F. Influence of oxygen on the proliferation and metabolism of adipose derived adult stem cells. *J Cell Physiol* 204: 184–191, 2005. [PubMed: 15754341]
40. Wang JS, Shum-Tim D, Chedrawy E, Chiu RC. The coronary delivery of marrow stromal cells for myocardial regeneration: pathophysiologic and therapeutic implications. *J Thorac Cardiovasc Surg* 122: 699–705, 2001. [PubMed: 11581601]
41. Wang Y, Haider HK, Ahmad N, Xu M, Ge R, Ashraf M. Combining pharmacological mobilization with intramyocardial delivery of bone marrow cells over-expressing VEGF is more effective for cardiac repair. *J Mol Cell Cardiol* 40: 736–745, 2006. [PubMed: 16603183]
42. Wisel S, Chacko SM, Kuppusamy ML, Pandian RP, Khan M, Kutala VK, Burry RW, Sun B, Kwiatkowski P, Kuppusamy P. Labeling of skeletal myoblasts with a novel oxygen-sensing spin probe for noninvasive monitoring of in situ oxygenation and cell therapy in heart. *Am J Physiol Heart Circ Physiol* 292: H1254–H1261, 2007. [PubMed: 17142337]
43. Zhao X, He G, Chen YR, Pandian RP, Kuppusamy P, Zweier JL. Endothelium-derived nitric oxide regulates postischemic myocardial oxygenation and oxygen consumption by modulation of mitochondrial electron transport. *Circulation* 111: 2966–2972, 2005. [PubMed: 15939832]
44. Zibaitis A, Greentree D, Ma F, Marelli D, Duong M, Chiu RC. Myocardial regeneration with satellite cell implantation. *Transplant Proc* 26: 3294, 1994. [PubMed: 7998137]

Fig. 1.

Electron paramagnetic resonance (EPR) and MRI images of hearts transplanted with labeled skeletal myoblast (SM) cells. *A*: slices of control hearts not implanted with cells or OxySpin. The EPR images contain uniform intensity (red) showing the contour of the heart. The grayscale MRI images show the heart sections. *B*: hearts implanted with OxySpin only. The EPR images show the distribution of OxySpin and no contour of the heart. The MRI images clearly show the site of implantation (arrows) of OxySpin in the heart. *C*: hearts transplanted with SM cells mixed (uninternalized) with OxySpin. The EPR images show the contour with a bright yellow spot indicating the presence of OxySpin at the site of implantation. The MRI images indicate the presence of OxySpin (arrows). *D*: hearts transplanted with SM cells labeled with OxySpin. The bright yellow spot in the EPR images show the OxySpin superimposed on the image of heart. The MRI images show the site as well as the spread of the probes in the left ventricular (LV) wall. A blank object was inserted into the LV for identification.

Fig. 2.

Representative EPR spectra of OxySpin in the heart. EPR spectra of a suspension of OxySpin in PBS equilibrated with room air (*A*) and 100% nitrogen (*B*). EPR spectra obtained from a mouse heart, in vivo, 1 day (*C*) or 2 wk (*D*) after implantation of OxySpin-labeled SMs. The peak-peak width (w) of the spectrum is a measure of oxygen concentration. The measured P_{O_2} values are noted on the respective spectrum.

Fig. 3.

Myocardial Po_2 in the infarcted heart at site of cell transplantation. Myocardial Po_2 values were measured repeatedly for 4 wk using in vivo EPR oximetry in mice hearts transplanted with OxySpin-labeled SM cells. *A*: tissue Po_2 at 4 wk after treatment with SM cells [myocardial infarction (MI) + SM] was significantly higher compared with untreated (MI) hearts. *B*: the time-course values of myocardial Po_2 measured from infarcted hearts (MI) and infarcted hearts treated with SM cells (MI + SM) are shown. Values are expressed as means $\pm$ SD ($n = 7$ animals/group). * $P < 0.05$ vs. MI group.

Fig. 4.

Functional assessment (echocardiography) of hearts at 4 wk after SM cell transplantation. Echocardiography was performed in noninfarcted hearts (Cont, control), infarcted hearts (MI), and infarcted hearts treated with SM cells (MI + SM). *Top*: representative recording of M-mode echocardiography. *Bottom*: LV ejection fraction, fractional shortening, and LV internal dimensions at the end systole (LVIDs) and end diastole (LVIDd), respectively. Results are expressed as means $\pm$ SD ($n = 6$ animals/group). * $P < 0.01$ vs. control; ** $P < 0.05$ vs. MI; # $P < 0.05$ vs. MI (LVIDd); ## $P < 0.05$ vs. MI (LVIDs).

Fig. 5.

Hemodynamic assessment of hearts at 4 wk after SM cell transplantation. The hemodynamic measurements were performed using a Millar catheter inserted through the right carotid artery into LV. *Top*: representative tracings of LV pressure (LVP) and rate of change of LVP (dP/dt) in noninfarcted hearts (control), infarcted hearts (MI), and infarcted hearts treated with SM cells (MI + SM). *Bottom*: hemodynamic data of the hearts at 4 wk after SM transplantation. HR, heart rate; bpm, beats/min; LVEDP, LV end-diastolic pressure; LVSP, LV systolic pressure; dP/dt_{max} and dP/dt_{min} , maximum rate of LVP increase and decline, respectively. Results are expressed as means $\pm$ SD ($n = 6$ animals/group). * $P < 0.01$ vs. control; ** $P < 0.05$ vs. MI.

Fig. 6.

Histological assessment of hearts after treatment with SMs. Sections show hematoxylin and eosin (H&E) and MY-32 (an immunohistochemical marker of myosin heavy chain) staining of tissue grafts at 1, 2, or 4 wk of postimplantation. *A* and *B*: hematoxylin and eosin and MY-32 staining of noninfarcted (control) heart sections ($\times 40$). *C* and *D*: hematoxylin and eosin and MY-32 staining showing the engraftment of SMs ($\times 40$) at 1 wk post-transplantation. *E*: hematoxylin and eosin staining ($\times 100$) showing the presence of engrafted cells at 2 wk posttreatment with SM. *F*: MY-32 staining ($\times 100$) showing positive staining (brown) for SMs at 2 wk posttreatment with SMs. *G*: MY-32 staining ($\times 400$) at 4 wk after transplantation showing the formation of myotubes. *H*: MY-32 staining of skeletal myocytes from a thigh muscle (positive control). Sk muscle, skeletal muscle.

Fig. 7.

Assessment of VEGF expression and capillary density in heart tissues harvested 4 wk after SM cell transplantation. Sections were stained with anti-VEGF, anti- α -smooth muscle actin (α -SMA), and anti-factor VIII antibodies to identify VEGF, blood vessels, and capillaries, respectively. Shown are fluorescence images of hearts stained with anti-VEGF (green) in untreated (MI; *A*) and treated (MI + SM; *B*) hearts, anti- α -SMA antibodies (red) in untreated (*C*) and treated (*D*) hearts, and anti-factor VIII antibodies (dark-red staining indicated by arrows) in the infarct zone of untreated (*E*) and treated (*F*), as well as in the

border zone of untreated (*G*) and treated (*H*) hearts. *C* and *D* also show counterstaining of nuclei using 4'-6-diamidino-2-phenylindole (blue). Also shown are quantitative assessment of VEGF expression (*I*) and the total number of capillaries in infarct and border zones (*J*). The capillary density assay was performed by counting the number of capillaries per high power field. AU, arbitrary units; HPF, high-power field. Results are expressed as means $\pm$ SD ($n = 6$ hearts/group). * $P < 0.05$ vs. respective MI group.

Fig. 8.

Assessment of infarct size and LV remodeling in hearts at 4 wk after SM cell transplantation. Infarct staining (*A*), infarct size (*B*), LV inner diameter (LV ID; *C*), and LV wall thickness (LV WT; *D*) are shown for noninfarcted hearts (control), infarcted hearts (MI), and infarcted hearts treated with SM cells (MI + SM). Values are expressed as means $\pm$ SD ($n = 6$ hearts/group). * $P < 0.01$ vs. control; ** $P < 0.05$ vs. MI.