Preclinical Evaluation of the Engineered Stem Cell Chemokine Stromal Cell–Derived Factor 1α Analog in a Translational Ovine Myocardial Infarction Model


Rationale: After myocardial infarction, there is an inadequate blood supply to the myocardium, and the surrounding borderzone becomes hypocontractile.

Objective: To develop a clinically translatable therapy, we hypothesized that in a preclinical ovine model of myocardial infarction, the modified endothelial progenitor stem cell chemokine, engineered stromal cell–derived factor 1α analog (ESA), would induce endothelial progenitor stem cell chemotaxis, limit adverse ventricular remodeling, and preserve borderzone contractility.

Methods and Results: Thirty-six adult male Dorset sheep underwent permanent ligation of the left anterior descending coronary artery, inducing an anteroapical infarction, and were randomized to borderzone injection of saline (n=18) or ESA (n=18). Ventricular function, geometry, and regional strain were assessed using cardiac MRI and pressure–volume catheter transduction. Bone marrow was harvested for in vitro analysis, and myocardial biopsies were taken for mRNA, protein, and immunohistochemical analysis. ESA induced greater chemotaxis of endothelial progenitor stem cells compared with saline (P<0.01) and was equivalent to recombinant stromal cell–derived factor 1α (P=0.27). Analysis of mRNA expression and protein levels in ESA-treated animals revealed reduced matrix metalloproteinase 2 in the borderzone (P<0.05), with elevated levels of tissue inhibitor of matrix metalloproteinase 1 and elastin in the infarct (P<0.05), whereas immunohistochemical analysis of borderzone myocardium showed increased capillary and arteriolar density in the ESA group (P<0.01). Animals in the ESA treatment group also had significant reductions in infarct size (P<0.01), increased maximal principle strain in the borderzone (P<0.01), and a steeper slope of the end-systolic pressure–volume relationship (P=0.01).

Conclusions: The novel, biomolecularly designed peptide ESA induces chemotaxis of endothelial progenitor stem cells, stimulates neovasculogenesis, limits infarct expansion, and preserves contractility in an ovine model of myocardial infarction. (Circ Res. 2014;114:650-659.)

Key Words: bioengineering ■ magnetic resonance imaging ■ myocardial infarction ■ translational research

Heart disease is the cause of significant morbidity and mortality in the United States, with estimates of ≈800,000 new acute coronary events each year and accounts for a substantial proportion of the national healthcare expenditure.1,2 The cellular events after a myocardial infarction (MI) result in inadequate microvascular perfusion that causes a change in the composition of the extracellular matrix (ECM), with regional alterations in levels of matrix metalloproteinase (MMP) and tissue inhibitors of metalloproteinase (TIMP). These pathophysiological changes result in hypocontractile scar formation, myocyte apoptosis, and progressive ventricular dilatation.3 The differential MMP/TIMP ratio leads to deleterious remodeling from proteolytic degradation of the ECM occurring over time. This increases the stress–strain relationship of ventricular myocytes and leads to inefficient contractility, infarct spreading, and ultimately heart failure.4–9 When current treatments for coronary artery disease fail, they usually

Original received October 21, 2013; revision received December 19, 2013; accepted December 23, 2013. In November 2013, the average time from submission to first decision for all original research papers submitted to Circulation Research was 14.6 days.

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The online-only Data Supplement is available with this article at http://circres.ahajournals.org/lookup/suppl/doi:10.1161/CIRCRESAHA.114.302884/-/DC1.

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Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/CIRCRESAHA.114.302884
do so because microvascular perfusion is not adequately restored—a critical, independent predictor of ventricular remodeling, reinfarction, heart failure, and death.10

Inadequate microvascular perfusion also plays an important role in the progression to heart failure after MI.10 To this end, many groups have experimented with various cell delivery and cytokine treatment regimens in an attempt to stimulate angiogenesis, with varying degrees of success.11–14 One such cytokine is stromal cell–derived factor 1α (SDF-1), which is a key regulator in hematopoietic stem cell trafficking between the bone marrow and peripheral circulation and effectively localizes endothelial progenitor cells (EPCs) to areas of ischemia.15–18 SDF-1 is a chemokine, stromal cell–derived factor 1α analog (SDF-1), that engages the 7 transmembrane domain G protein–coupled receptor CXCR 4. After MI, cardiac secretion of SDF-1 is insufficiently robust to mobilize a sufficient population of EPCs to affect meaningful changes in perfusion. However, supraphysiologic doses of intramyocardial SDF-1 have been shown by our group and others to increase vasculogenesis, decrease cardiac myocyte apoptosis, increase cardiac myocyte survival, and preserve ventricular geometry.19–25 Despite the reported beneficial effects of recombinant SDF-1 after an ischemic insult, it has some limitations. SDF-1 is broken down by multiple peptidases (CD26/DPP-IV) and is chemically modified, synthetic version of SDF-1, which we named engineered stromal cell–derived factor 1α analog (ESA), to overcome these hurdles, and have shown it to be effective in a small animal model of MI.26–31 In the current study, we hypothesized that intramyocardial delivery of ESA in a small animal model of MI would result in chemotaxis of EPCs, increased microvascularization, limited ventricular remodeling, and improved regional and global ventricular function.

Methods

All experiments pertaining to this investigation conformed to the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (Eighth Edition, 2011). The protocol was approved by the Institutional Animal Use and Care Committee of the University of Pennsylvania (protocol number 803430).

Custom Peptide Synthesis

We have previously reported on the design and synthesis of ESA.27 Briefly, to minimize the profile of the peptide, the CXCR4 receptor binding N-terminus and the molecular stabilizing C-terminus were preserved, whereas the central β-sheet pleated sheet was deleted. Using computational modeling, it was determined that a 2-proline residue linker joining this modified sequence would durably retain a 3-dimensional (3D) protein configuration similar to the native SDF-1 (Online Figure I). The engineered protein was then synthesized using solid-phase peptide synthesis, where the N-α-amino acids were incorporated into the peptide in a stepwise fashion while the C-terminal end was attached to a solid support matrix.

Endothelial Progenitor Cell Chemotaxis

Bone marrow mononuclear cells were isolated from the long bones of adult male Dorset sheep by density centrifugation with Histopaque 1083 (Sigma-Aldrich), plated on vitronectin-coated dishes, and cultured in endothelial basal medium 2 supplemented with EGM-2 SingleQuot (Lonza) containing human epidermal growth factor, fetal bovine serum, vascular endothelial growth factor, basic human fibroblast growth factor, recombinant human long R3 insulin-like growth factor 1, ascorbic acid, heparin, gentamicin, and amphotericin-B. Media was changed on culture day 4, and nonadherent bone marrow mononuclear cells were discarded, enriching for the EPC phenotype. A modified transwell migration assay (Boyden Chamber, Neuro Probe, Gaithersburg, MD) was used to assess EPC migration as previously described (see online-only Data Supplement for a detailed description).21,26,27

Animal Model

MI was induced in 36 adult male Dorset sheep (weighing between 35 and 45 kg) using an established and highly reproducible model.15 Briefly, the animals were induced with intramuscular midazolam (0.4 mg/kg) and ketamine (5 mg/kg), and anesthesia was maintained on inhaled isoflurane (1.5%–3%). An anterior, 5 cm mini-thoracotomy was used to access the left chest cavity, and the heart was exposed. The distal left anterior descending and the second diagonal coronary arteries were ligated with a 4-0 polypropylene suture, creating an anteroseptal area of ischemia (Figure 1). Animals were randomized to receive either 1 mL of saline (n=18) or 6 μg/kg of ESA (n=18) diluted in 1 mL of saline injected via a custom-made 25-gauge end-capped needle (Cadence Inc, Staunton, VA) with 3 side holes each separated by 120° in 10 equal aliquots around the borderzone of the ischemic myocardium. The concentration of ESA administered was chosen based on dose response curves on which we have previously published.26,29 Continuous ECG monitoring was used throughout the procedure. All animals received an intramuscular injection of sargramostim (granulocyte-macrophage colony-stimulating factor, 40 μg/kg). Animals were recovered from anesthesia, and buprenorphine (0.05 μg/kg IM) along with a transdermal fentanyl patch (75 μg/h) was used for postoperative pain control. A total of 5 animals developed refractory ventricular fibrillation immediately after ligation (n=3 in the saline group, n=2 in the ESA group), and 1 animal in the saline group died during the follow-up period (autopsy revealed intestinal volvulus with resultant perforation). All surviving animals were euthanized 8 weeks after MI.

Cardiac MRI

A subset of animals (ESA, n=7; saline, n=5) underwent cardiac MRI before and 8 weeks after MI to assess global ventricular function, ventricular geometry, and 3D strain. General anesthesia was maintained for the entirety of the imaging procedures as described above. A high-fidelity pressure transduction catheter (Millar Instruments, Houston, TX) was inserted into the left ventricle (LV) via the carotid artery under fluoroscopic guidance for LV pressure gating during image acquisition. MRI was performed using a 3T MAGNETOM Verio scanner (Siemens, Malvern, PA) before infarction and 8 weeks after infarction as previously described (see

Nonstandard Abbreviations and Acronyms

<table>
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<th>Abbreviation</th>
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<tr>
<td>3D</td>
<td>3-dimensional</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<td>EF</td>
<td>ejection fraction</td>
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<td>EPC</td>
<td>endothelial progenitor cell</td>
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<td>ESA</td>
<td>engineered stromal cell–derived factor 1α analog</td>
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<td>LV</td>
<td>left ventricle</td>
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<td>MI</td>
<td>myocardial infarction</td>
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<td>MMP</td>
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<td>PV</td>
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<td>SDF</td>
<td>stromal cell–derived factor</td>
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<td>TIMP</td>
<td>tissue inhibitor of matrix metalloproteinase</td>
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online-only Data Supplement for a detailed description and Online Movie I for representative long-axis cine loops).33

MR Image Postprocessing

Treatment groups of the imaging data sets were blinded to the end-user throughout postprocessing. LV volume and global function data were obtained using prospective steady state free precession cine MR images. Raw short-axis images were automatically sorted, cropped, and contrast normalized in a custom Matlab (Natick, MA) program to ensure homogeneous LV coverage and image quality, respectively. Segmentation was then performed through all cardiac phases of the sorted and correct images using a semi-automated 3D active contour segmentation program (ITK-SNAP, open access/source).34 LV end-diastolic volume, end-systolic volume, stroke volume, and ejection fraction (EF) were then computed throughout the entire cardiac cycle from segmented images using in-plane and through-plane spatial resolution information. Systolic regional strain measurements from the borderzone were calculated from 3D spatial modulation of magnetization acquisitions using an optical flow technique as previously described (Figure 2).35 Maximum principle strain ($\varepsilon_1$) was then calculated for each region, and 3D strain maps were generated (see online-only Data Supplement for a detailed description).

Invasive Hemodynamic Assessment

Pressure–volume (PV) loops were acquired in all animals before infarction and 8 weeks after MI. A Ventri-Cath PV catheter transducer (Millar Instruments Inc, Houston, TX) was placed into the LV via the right carotid artery. Steady state PV loops were acquired during a 10-second breath hold, followed by inferior vena cava occlusion to produce static and dynamic loops under varying load conditions. Coronary angiograms were performed, and cardiac output was measured using periaortic Doppler flow probes (Transonic Systems, Ithaca, NY) (see online-only Data Supplement for a detailed description and Online Movie II for a representative coronary angiogram).

Histological Analysis and Immunohistochemistry

To assess ventricular geometry, infarct size, and microvascular angiogenesis, hearts were explanted after the invasive hemodynamic assessment was performed and flushed with saline. The atria were removed, and the LV opened along the posterior septum. Standardized digital photographs were taken with a Nikon D5100 SLR camera (Nikon, Tokyo, Japan). Photographs were uploaded to ImageJ (v1.46b) and the size of the infarct assessed with digital planimetry. Ventricular samples (3 cm$^3$) were then calculated from the infarct, borderzone, and remote myocardium, snap frozen in liquid nitrogen, and stored in a −80ºC freezer. Ten-micrometer-thick sections were prepared from each biopsy specimen. Ten-micrometer-thick sections from the borderzone of each heart were stained with antibodies directed against von Willebrand Factor and separately for $\alpha$-smooth muscle actin to quantify capillary and arteriolar density as previously described.31

Ventricular ECM mRNA Profiling

RNA was isolated from frozen myocardium using a QuantiGene Sample Processing kit (Affymetrix) according to the manufacturer’s instructions.
mRNA was analyzed with a MMP profile (1, 2, 7, 8, 9, 12, 14), TIMP profile (1, 2, 4), elastin, and transforming growth factor-β using QuantiGene 2.0 Plex Assay kit (Affymetrix) according to the manufacturer’s protocol (see online-only Data Supplement for detailed description).

Ventricular ECM Protein Expression
Myocardial biopsies (10 mm³) were removed from the LV at the time of explant (regions: infarct, borderzone, and remote) and snap frozen in liquid nitrogen. Samples were homogenized in T-PER protein extraction reagent along with HALT protease inhibitor (Thermo Scientific, Waltham MA). Final protein concentrations were determined using the Qubit protein assay (Qubit fluorometer, Life Technologies, Carlsbad CA). The extracellular membrane profile of each myocardial region was assessed via immunoblotting (see online-only Data Supplement for further details including specific antibodies used).

Statistical Analysis
All analyzed variables approximated a normal distribution, and values for continuous variables were reported as mean±SD. A 1-way ANOVA with Tukey correction was used for comparison of continuous variables between groups. Statistical significance was set at P<0.05. Analyses were performed with STATA (StataCorp, College Station, TX) statistical software package, version 12.1.

Results
ESA Induces Chemotaxis of Sheep EPCs
To confirm the efficacy of ESA in sheep, a transwell migration assay was used. EPCs showed significantly increased chemotaxis toward ESA compared with saline alone (saline, 97.3±4.9 EPCs/mm²; ESA, 611±21.7 EPCs/mm²; P<0.01), indicating that the custom peptide actively binds to sheep EPCs. Importantly, the degree of EPC chemotaxis was similar between the ESA and SDF-1 groups (SDF, 568±37.5 EPCs/mm²; ESA, 611±21.7 EPCs/mm²; P=0.27; Figure 3).

Differential Ventricular ECM mRNA Expression and Protein Concentration
Biopsies from the infarct, borderzone, and remote myocardium were taken for RNA analysis and assessed for MMP, TIMP, elastin, and transforming growth factor-β levels. RNA levels of MMP-2 were found to be elevated in the borderzone myocardium from sheep in the saline control group compared with the ESA group (saline, 0.1±0.01 ratio of fluorescence unit [RFU]; ESA, 0.03±0.01 RFU; P<0.05), whereas there was no difference in the other MMPs from any myocardial region between groups. Interestingly, RNA levels of TIMP-1 and elastin were elevated in the infarct of ESA-treated animals (TIMP-1; saline, 0.3±0.4 RFU; ESA, 2.4±1.0 RFU; P<0.05, elastin, saline, 0.3±0.2 RFU; ESA, 3.1±1.2 RFU; P<0.05). RNA expression profiles from all regions are presented in Figure 4.

To determine whether the differential changes in MMP and TIMP mRNA expression between groups led to differences in protein concentration and were not undergoing alterations in post-transcriptional or post-translational modification events, immunoblots were performed. Results were consistent with the mRNA expression data, where protein levels of MMP-2 were elevated in the myocardium of sheep from the saline group compared with the ESA group (saline, 0.58±0.05; ESA, 0.074±0.037; P<0.05), with no differences in any of the other MMP protein levels between groups. Again, TIMP-1 and elastin protein concentrations were higher in the infarct region of myocardium from ESA-treated animals (TIMP-1; saline, 0.38±0.03; ESA, 0.73±0.18; P<0.05, elastin, 0.17±0.04; ESA, 0.66±0.3; P<0.05). There were no significant differences among the other protein levels of myocardial regions between groups. Representative immunoblot results for MMP-2 (borderzone), TIMP-1 (infarct), and elastin (infarct) are presented in Figure 4.

Microvascular Density and Myocardial Perfusion
Biopsies from the borderzone were sectioned and stained for von Willebrand Factor and α-smooth muscle actin. Analysis of immunofluorescently labeled von Willebrand Factor and α-smooth muscle actin showed a significant increase in both capillary and arteriolar density in the ESA group compared with the saline control (von Willebrand Factor: saline, 7.5±1.4 capillaries/mm²; ESA, 17.7±4.2 capillaries/mm²; P<0.01; smooth muscle actin: saline, 10.8±2.6 arterioles/mm²; ESA,
8 weeks after MI. 

To determine if the increased vascularity actually improved myocardial perfusion, coronary angiograms were performed and assessed for collateral filling using a previously validated method described by Rentrop et al.\(^36\) Rентrop scores were calculated for each animal and averaged for each group. The saline group had significantly less collateral filling compared with the ESA group (saline, 0.5±0.7; ESA, 2.0±0.8; \(P<0.05\)) (see online-only Movie II for a representative coronary angiogram).

### ESA Treatment Improves Regional Strain and LV Function While Limiting Remodeling

Maximal principle strain (\(\varepsilon_1\)) was measured in the borderzone region of animals treated with ESA and saline before and 8 weeks after MI. \(\varepsilon_1\) is indicative of radial myocardial displacement, and the magnitude of in-plane displacement was depicted as a color map overlay (Figure 6). Late gadolinium enhanced images were used for infarct identification and aided in choosing the appropriate regions of interest within the strain color maps. The composite strain data show that animals treated with ESA had significantly elevated \(\varepsilon_1\) within the borderzone compared with saline control (saline, 8.8±3.3%; ESA, 12.4±5.8%; \(P<0.01\)), indicative of differential systolic stretching and radial wall thickening between groups. Because radial strain was measured, changes in the thickness of borderzone myocardium between systole and diastole could be evaluated, measures that correlate to regional contractility. Thus, the increased borderzone strain seen in hearts from animals in the ESA group is a result of improved and more efficient myocardial contraction compared with hearts from animals in the saline group.

Steady state free precession cine MR images were used to calculate EF, stroke volume, end-systolic volume, and end-diastolic volume for both treatment groups. At baseline, the EF was 46%, similar to previous studies using Dorset sheep.\(^{27,38}\) Eight weeks after infarction, animals in the saline group had a decline in EF to 27.8±4.9%, whereas animals in the ESA group had a relatively preserved EF of 36.4±6.4% (\(P=0.01\)). In addition, animals in the saline group had significantly larger end-systolic and end-diastolic volumes (end-systolic volume: saline, 96.5±21.2 mL; ESA, 70.4±12.4 mL; \(P=0.01\); end-diastolic volume: saline, 136.9±22.4 mL; ESA, 109.3±17.5 mL; \(P<0.05\)), suggestive of reduced ventricular dilatation and attenuation of LV remodeling in the ESA group (Figure 7).
LV systolic function was further assessed using a PV catheter transducer and aortic flow probe. The slope of the end-systolic PV relationship curve was derived from PV loops during inferior vena cava occlusion and represents end-systolic elastance, an approximation of myocardial contractility (see Figure 7 for representative PV loops). Animals in the ESA group had an elevation in the slope of the end-systolic PV relationship compared to animals in the saline group (saline, 1.6±0.07; ESA, 6.9±2.6; \( P=0.01 \)), consistent with improved LV contractility. Furthermore, cardiac output, as measured by periaortic flow

**Figure 5. Vascular density and myocardial perfusion.** Immunohistochemical light microscope images of borderzone myocardium using anti–von Willebrand Factor (vWF) antibody (A and B) and anti–α-smooth muscle actin (αSMA) antibody (C and D). Analysis of immunofluorescent expression of vWF and αSMA revealed a significant increase in signal in the engineered stromal cell–derived factor 1α analog (ESA) group compared with saline (E and F, respectively). Coronary angiograms were reviewed and assessed for collateral filling using Rentrop scores in an effort to evaluate whether or not the increased vascularity seen from the immunohistochemical data resulted in increased myocardial perfusion. G, Coronary angiograms from animals in the ESA group had significantly higher Rentrop scores, and hence better collateral filling, compared with angiograms from animals in the saline group (\( P<0.05 \); scale bar=75 μm). DAPI indicates 4’,6-diamidino-2-phenylindole.

**Figure 6. Maximal principle strain analysis.** A, Color map of regional maximum principle strain from cross-sections of the left ventricle (LV). Representative color maps of hearts from engineered stromal cell–derived factor 1α analog (ESA)–treated animals show elevated (red on color map) regional strain about the borderzone. Hearts from saline-treated animals show decreased (green to blue) regional strain about the borderzone. Representative color map of a heart from a normal animal without myocardial infarction shows a characteristic strain pattern with elevated strain throughout the free wall of the LV. B, Cross-sectional late gadolinium enhanced MR images corresponding to the strain maps from the saline and ESA groups (white arrows delineate boundary of infarct; scar tissue shows up white from gadolinium contrast). C, Regional strain at the borderzone is significantly elevated in hearts from animals treated with ESA compared with hearts from animals treated with saline and closer to healthy myocardium (\( P<0.05 \)).
probe assessment, was significantly higher in the ESA group compared with the saline group (saline, 2.8±0.8 L/min; ESA, 4.1±1.1 L/min; \( P = 0.01 \)). There was no difference in heart rate between groups.

At the time of terminal surgery, after all invasive hemodynamic parameters were obtained, animals were euthanized with retrograde KCl injection, hearts were explanted, the LV was opened, and the infarct was outlined and photographed. Quantification of infarct size, measured as a percentage of total LV area, revealed a significant infarct reduction in the ESA group compared with the saline group (saline, 21±1.9%; ESA, 14±0.4%; \( P < 0.01 \); Figure 8).

**Discussion**

In this study, we attempted to evaluate the efficacy of intramyocardial injection of the novel engineered peptide ESA in a large animal model of MI to demonstrate the translational potential of this peptide. Here, we have successfully shown that ESA treatment results in effective EPC chemotaxis, increased capillary density in the borderzone, elevated borderzone maximal principle strain, reduced LV remodeling, and improved LV dynamics in post-MI sheep. These data build on our initial experience with ESA in small animal models of MI, where similar findings were noted.\(^{26,27,30}\) Initially, we attempted to show that ESA retained its chemokine effect on sheep EPCs because we had previously demonstrated its efficacy on only mouse and rat EPCs. The results from the transwell migration assay conclusively prove the ability of ESA to induce migration of sheep EPCs. This holds significant importance when evaluating capillary and arteriolar density between the ESA and saline groups because ESA localizes EPCs to the site of injury, initiating neovasculogenesis and creating new capillaries resulting in perfused arteriolar networks. The microvascularization that takes place after ESA treatment allows for retention of the ECM, reduction of infarct spreading, and improved regional and global LV function.

To further understand the mechanism behind the attenuation of LV remodeling seen in the ESA group, we studied the transcriptional regulation of select MMPs/TIMPs by quantification of mRNA levels within different regions of the ventricle (infarct, borderzone, and remote) and further assessed myocardial samples for their protein concentrations, showing concordance between mRNA and protein expression. Other groups have clearly shown that MMPs are upregulated in the infarct and borderzone after MI (specifically MMP 1, 2, 13, and 14), whereas TIMPs are downregulated (specifically TIMP 1, 2, and 4) compared with levels found in

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Figure 7. Hemodynamic assessment. Pressure–volume (PV) loops were obtained during inferior vena cava (IVC) occlusion for variable load data. A and B, Representative PV loops from animals in the saline group and engineered stromal cell-derived factor 1α analog (ESA) group. Note the increased slope of the end-systolic PV relationship (ESPVR; red line) in the ESA group, a preload independent measure of myocardial contractility. C, Before PV loop acquisition, a small upper midline laparotomy was performed, and the suprahepatic IVC was dissected free and encircled with a vessel loop. This is a representative image of complete IVC occlusion by gentle retraction on the vessel loop. D, Animals in the ESA group had improved ejection fraction (EF), lower end-systolic volume (ESV), and lower end-diastolic volume (EDV) compared with animals in the saline group, representing preserved LV function and limited LV remodeling (*\( P < 0.05 \)). SV indicates stroke volume.

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normal myocardium.\textsuperscript{6–8} This regional and type specific alteration in the MMP/TIMP expression profile leads to dysfunctional alteration in LV geometry because of ECM breakdown and infarct expansion. Our findings of decreased expression of MMP-2 within the borderzone and increased expression of TIMP-1 within the infarct of ESA-treated animals compared with saline control provide causal support for the decreased infarct size and limited LV remodeling seen within the ESA group. We suspect that the chemotactic properties of ESA localize EPCs to and around the infarct, causing microvascularization, leading to preservation of the ECM and differential expression of MMPs and TIMPs, respectively. This theoretical explanation is supported by a study showing increased TIMP-1 and TIMP-2 levels in vitro from secretion by CD34+ bone marrow progenitor cells.\textsuperscript{39} It is also worth mentioning that elastin mRNA and protein levels within infarcted tissue were statistically higher in the ESA group, an additional finding that supports maintenance of ECM within the infarct. The benefits of increased elastin levels within an infarct, such as decreased remodeling and improvement in LV hemodynamics, has been published.\textsuperscript{40,41}

A key innovation in the current study was the application of highly advanced postprocessing software for the calculation of regional strain relying on sophisticated 3D cardiac MRI tissue tagging technology using a 3D optical flow technique. This externally validated method has proven enormously useful in the ability to assess myocardial strain accurately in vivo, and with excellent in-plane and through-plane resolution, accurate quantification of regional strain is possible. Our results of increased regional strain in the borderzone of ESA-treated animals suggest that myocardium in this region is thicker at end systole, which may be indicative of improved regional contractility. This is an intriguing finding and one that we have hypothesized in the past, but until now we have been unable to definitively prove.\textsuperscript{30} With increased borderzone strain, ESA-treated animals exhibited enhanced global LV contractility with a significant elevation in the slope of the end-systolic PV relationship along with greatly improved cardiac output. The beneficial effects ESA has on LV function after MI is likely multifactorial and begins with increased borderzone vascularity, which leads to limited expansion of the infarct and decreased LV remodeling. The preservation of regional strain within the borderzone and its effect on global LV function have important clinical implications for the use of ESA in the prevention of ischemic cardiomyopathy in patients with acute MI.

Limitations

One limitation of this study is that it was performed in an acute animal model of MI, where the treatment was given immediately after coronary artery ligation. Although this satisfies our question for how ESA interacts with bone marrow progenitor cells and the effects these cells have on the myocardium, the positive findings reported here may not be extrapolated to include the breadth of pathology seen in human subjects, as often times treatment modalities must be effective in the setting of chronic heart failure. Chronic postischemic changes in a typical patient with ischemic cardiomyopathy offer a complex problem to the investigator, where deleterious remodeling has already taken place and a diminutive stem cell supply must be overcome. However, as a proof of principle study and a starting point, we have demonstrated here that ESA is effective in a sheep model of MI.
Conclusions
In summary, intramyocardial injection of ESA induces chemotaxis of EPCs resulting in microrevascularization of the myocardium, increases maximal principle strain in the borderzone, limits ventricular remodeling, and improves ventricular function. The findings presented here illustrate the efficacy of ESA therapy post-MI and provide substantial support for the translational potential of this novel peptide in the treatment of heart disease.

Acknowledgments
We thank Jean Boyer (University of Pennsylvania Immunology core laboratory) for her help and expertise with myocardial RNA isolation and analysis and Gerald Zsido II (University of Pennsylvania, Gorman Cardiovascular Research Group) for his guidance and expert assistance with cardiac gated MRI acquisition.

Sources of Funding
This study was supported, in part, by National Institutes of Health (NIH) grants R01 HL089315-01, Angiogenic Tissue Engineering to Limit Post-Infarction Ventricular Remodeling (Y.J. Woo); Post-Doctoral Fellowship Grant from the American Heart Association (H.J. Gorman); Established Investigator Awards from the American Heart Association (R. C. Gorman, J. H. Gorman); and a Scientist Development Grant from the American Heart Association (P. Attiluri).

Disclosures
None.

References


**Novelty and Significance**

**What Is Known?**

- Delivery of bone marrow progenitor cells after a myocardial infarction (MI) improves ventricular function.
- Stromal cell-derived factor 1α (SDF-1) mobilizes endothelial progenitor cells (EPCs) from the bone marrow to areas of myocardial ischemia; however, cardiac secretion of SDF-1 is insufficiently robust to mobilize a sufficient number of EPCs to affect meaningful changes in perfusion.
- SDF-1 is broken down by multiple peptidases, and recombinant production is inefficient and expensive.

**What New Information Does This Article Contribute?**

- Using computational modeling, a biomolecularly designed version of SDF-1 was created to overcome the limitations of the recombinant protein.
- Engineered SDF-1α analog (ESA) effectively recruits EPCs.
- Intramyocardial delivery of ESA in a preclinical, large animal acute MI model, leads to increased myocardial perfusion, smaller infarcts, improved borderzone contractility, and limited ventricular remodeling.
- After an MI, the resulting deficit in microvascular perfusion ultimately leads to myocyte apoptosis, infarct spreading, ventricular dilatation, and heart failure. Localization of EPCs to the border zone of the infarct preserves myocardial perfusion and limits myocardial injury. Cardiac secretion of SDF-1 is unable to recruit a sufficient number of EPCs to achieve improved perfusion. We hypothesized that ESA delivery to the infarct border zone would induce EPC chemotaxis, increase myocardial perfusion, limit ventricular remodeling, and enhance borderzone contractility. We found clear EPC chemotaxis across an ESA gradient and significant regional functional improvements in ventricular function in a large animal, acute MI model. Preservation of LV function is due to the myocardial microvascularization caused by ESA treatment, resulting in reduced ventricular remodeling. Together, these findings provide support for the translational use of ESA in human subjects with acute MI in an effort to prevent the progression to ischemic cardiomyopathy.
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_Circ Res._ 2014;114:650-659; originally published online December 23, 2013;
doi: 10.1161/CIRCRESAHA.114.302884

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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SUPPLEMENTAL MATERIALS

Supplemental Methods

Endothelial Progenitor Cell Chemotaxis

A modified transwell migration assay (Boyden Chamber, Neuro Probe, Gaithersburg, MD) was used to assess EPC migration. 8-µm filters were loaded into control and experimental chambers. Seven-day EPCs cultured in endothelial-specific media on vitronectin-coated plates were trypsinized, counted, and brought to a concentration of 90 cells/µl in Dulbecco’s phosphate buffered saline (DPBS). The bottom chamber was loaded with either 100ng/ml of recombinant SDF-1 (R&D Systems, Minneapolis, MN) in DPBS or 100ng/ml of ESA in DPBS. Saline alone served as a control. A 560µl cell suspension was added to the top chamber of each well. All chambers were incubated at 37ºC, 5% CO₂ for 3.5 hours. The cells remaining in the top chamber were wiped clean with a cotton swab and the filter was removed, placed on a glass slide, and mounted with Vectashield + DAPI (Vector Laboratories). Slides were visualized on a DF5000B Leica fluorescent microscope and cell density was analyzed via LASAF version 2.0.2 (Leica; Wetzlar, Germany) software. Boyden chamber analyses were performed in triplicate.

Cardiac Gated MRI Image Acquisition

General anesthesia was maintained for the entirety of the imaging procedures, as described above. A high-fidelity pressure transduction catheter (Millar Instruments; Houston, TX) was inserted into the left ventricle (LV) via the carotid artery under fluoroscopic guidance for LV pressure gating during image acquisition. MRI was performed using a 3T MAGNETOM Verio scanner (Seimens; Malvern, PA) before infarction and 8 weeks after infarction. Animals underwent prospectively-gated 3D SSFP cine MRI for volumetric analysis using the following parameters: field of view – 300 x 244, acquisition matrix – 192 x 156, repetition time – 3.11ms, echo time – 1.53ms, bandwidth – 1184Hz/pixel, slice thickness – 4mm, averages – 2. Regional borderzone LV strain was assessed using a 3D spatial modulation of magnetization (SPAMM) tagged sequence with the following parameters: field of view – 260 x 260, acquisition matrix – 256 x 128, repetition time – 34.4ms, tag spacing – 6mm, bandwidth 330Hz/pixel, slice thickness – 2mm, averages – 4.¹ Fifteen minutes following intravenous injection of 0.1mmol/kg gadobenate dimeglumine (MultiHance; Bracco Diagnostics, Princeton, NJ), infarct location and wall thickness were visualized using a 3D late-gadolinium enhanced (LGE) spoiled gradient echo sequence with the following parameters: field of view – 350 x 350, acquisition matrix – 256 x 256, repetition time – 591.28ms, echo time – 2.96ms, inversion time – 200-300ms, flip angle - 25°, averages – 2. Images were archived and stored off-line for post-processing.

Calculation of Regional Strain Using the Optical Flow Technique

Systolic regional strain measurements from the borderzone were calculated from 3D SPAMM acquisitions (Figure 2) using an optical flow technique.² Epicardial and endocardial contours were manually segmented at the end diastolic reference state using ImageJ software (NIH; Bethesda, MD). Image masks were created from segmented contours to isolate LV myocardium. A custom optical flow plug-in for ImageJ was used to derive the x, y, and z displacement flow fields from the stack of tagged images through end-systole. Using a custom Matlab program, infarct boundaries were identified using LGE landmarks. Borderzone myocardium was automatically defined by the region extending 20 degrees beyond the infarct
boundaries on each side. Remote regions were automatically defined as the regions extending 40 degrees beyond the borderzone boundaries on each side. The endocardial and epicardial edges were excluded to reduce noise from blood and lung movement, respectively. Maximum principle strain ($\varepsilon_1$) was then calculated for each region and 3D strain maps were generated.

**Invasive Hemodynamic Measurements**

At the start of each surgery, a 14 gauge single lumen catheter was inserted into the right femoral artery for continuous blood pressure monitoring, and a pulmonary artery catheter was placed for continuous cardiac output and central venous pressure monitoring. Pressure volume (PV) loops were acquired in all animals before infarction and 8 weeks following MI. A ventricath PV catheter transducer (Millar Instruments Inc, Houston, TX) was zeroed in saline before placement into the LV via the right carotid artery. The suprahepatic inferior vena cava (IVC) was encircled with a vessel loop. Steady state PV loops were acquired during a 10 second breath hold, followed by IVC occlusion in order to produce static and dynamic loops under varying load conditions. Data were recorded and analyzed with the MPVS Ultra PV System (Millar Instruments, Inc) and LabChart version 7.3.1 software (AD instruments). At the time of terminal surgery, animals underwent coronary catheterization and PV loops were acquired as above. This was followed by sternotomy and cardiac output was assessed via peri-aortic Doppler flow probe (Transonic Systems, Ithaca, NY) placement around the ascending aorta. The aortic root was cannulated, and 40 mEq of potassium chloride was injected in a retrograde fashion to induce cardiac arrest. Hearts were explanted for analysis.

**Coronary Angiogram**

At the time of terminal surgery, animals were induced with IM midazolam (0.4mg/kg) and ketamine (5mg/kg), an endotracheal tube was inserted, and anesthesia was maintained on inhaled isoflurane (1.5-3%). A 5cm skin incision was made in the right neck, and the right carotid artery was dissected free, proximal and distal control was achieved with vessel loops, and a 6 french (Fr) sheath introducer (Cordis, Bridgewater NJ) was inserted into the carotid artery and secured. An EMERALD PTFE coated straight tip 0.035in guidewire (Cordis) was inserted into the aortic root under fluoroscopic guidance, over which a JL4 catheter (Cordis) was advanced into the left coronary os. 10cc of Visipaqe (GE Healthcare) was injected and angiograms were taken and saved on an RS Axiom Artis Silver universal floor mounted C-arm angiography system (Siemens Medical Solutions, Malvern PA). Angiograms were evaluated for collateral flow by a blinded observer. A representative coronary angiogram from an ESA treated animal can be viewed in the Online Supplemental Video II.

**Immunohistochemistry**

10µm thick sections from the borderzone of each heart were stained with antibodies directed against von Willebrand Factor (vWF) and separately for alpha smooth muscle actin ($\alpha$SMA) in order to quantify capillary and arteriolar density, respectively. Sections were fixed with ice cold acetone, blocked in 10% FBS, and incubated with either rabbit anti-vWF (1:200 dilution; Abcam) or mouse anti-$\alpha$SMA (1:50 dilution; Thermo) for 2 hours. Donkey anti-rabbit antibody (Alexa Fluor 488, 1:200; Abcam) or donkey anti-mouse antibody (Alexa Fluor 555, 1:50 dilution; Invitrogen) were used as secondary reagents and incubated for 1 hour, after which sections were washed and counterstained with DAPI to visualize nuclei. Quantitative analysis of capillary and arteriolar density within the borderzone was conducted under the 20x objective
of a Leica DM5000B microscope. Group blinded counts were averaged over 4 fields per specimen.

**Ventricular Extracellular Matrix mRNA Profiling**

RNA was isolated from frozen myocardium using a QuantiGene Sample Processing kit (Affymetrix) according to the manufacturer's instructions. Briefly, each biopsy specimen was added to 300μl of homogenizing solution with 6μl of proteinase K and was then incubated at 65°C for 1-2 hours while vortexing every 10 minutes. After centrifugation, the supernatant was analyzed with a matrix metalloproteinase profile (1,2,7,8,9,12,14), tissue inhibitor of matrix metalloproteinase profile (1,2,4), elastin, and transforming growth factor beta using QuantiGene 2.0 Plex Assay kit (Affymetrix) according to the manufacturer's protocol. Forty μl of tissue supernatant was added to each well of the hybridization plate containing 60μl of working bead mix and was incubated for 18-22 hours at 54°C at 600rpm. Forty μl of homogenizing solution was used for assay background. The samples were transferred to the magnetic separation plate followed with pre-amplifier hybridization, amplifier hybridization, probe labeling, and SAPE binding with three washes after each step. Lastly, the plate was analyzed in the Bio-Plex Luminex 200 system (BIO-RAD), gene expression was calculated using Bio-Plex Manager 5.0 software, and results were standardized to GAPDH expression. Results are reported in ratio of fluorescence units (RFU).

**Ventricular Extracellular Matrix Protein Expression**

LV myocardial biopsies (10mm3) were removed from the LV at the time of explant (regions: infarct, borderzone, and remote) and snap frozen in liquid nitrogen. Samples were homogenized in T-PER protein extraction reagent along with HALT protease inhibitor (Thermo Scientific, Waltham MA). Final protein concentrations were determined using the Qubit protein assay (Qubit fluorimeter, Life Technologies, Carlsbad CA). The extracellular membrane profile of each myocardial region was assessed via immunoblotting. The Novex NuPAGE SDS-PAGE Gel system (Life Technologies, Carlsbad CA) was employed to electrophorese protein samples on 4-12% 1.5 mm Bis-Tris SDS-polyacrylamide gels. Protein were subsequently transferred to PVDF membranes using the iBlot dry transfer method (Life Technologies, Carlsbad CA) with 0.2 μm pore size at 20V. Membranes were blocked by immersion for 1 hour in TBS-0.1% Tween (TBST) with 5% nonfat dry milk (NFDM). All antibodies were diluted in TBST-5%NFDM (Online Table I). After protein separation and transfer, membranes were incubated with antibodies against an MMP/TIMP panel along with TGF-β and elastin by incubation with the corresponding secondary antibody. A chemiluminescence kit (Luminata Western HRP Substrates, Millipore, Billerica MA) was used for immunodetection, and bands were imaged using a Image Quant LAS 4000 system (GE Healthcare, Little Chalfont UK). All membranes were stripped after immunodetection and re-probed with anti-β actin (Abcam, Cambridge UK). The ratio of protein to actin was used to quantify protein levels between groups.
Online Figure I.

Ribbon Diagram. The beta pleated sheet in SDF has been deleted in ESA, and the N and C termini have been joined by two proline residues.

Online Table I. Primary Antibody Information

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References


Online Video I. **Cardiac Gated MRI, Cine Loops.** Representative two chamber, long axis cine loops are displayed from an animal without an infarct (Normal), an animal from the saline group (Saline), and an animal from the ESA group (ESA). There is reduced LV function in both the saline and ESA treated hearts; however the ESA treated heart has relatively preserved function.

Online Video II. **Coronary Catheterization.** Left coronary angiogram from an animal in the ESA group. There is complete occlusion of the LAD towards the apex, with significant collateral flow from the diagonal and circumflex coronary arteries.