Improvement in Cardiac Function With Small Intestine Extracellular Matrix Is Associated With Recruitment of C-Kit Cells, Myofibroblasts, and Macrophages After Myocardial Infarction

Zhi-Qing Zhao, MD, PhD,* John D. Puskas, MD,† Di Xu, MD, † Ning-Ping Wang, MD,* Mario Mosunjac, MD, † Robert A. Guyton, MD, † Jakob Vinten-Johansen, PhD, † Robert Matheny, MD‡

Savannah and Atlanta, Georgia

Objectives This study tested the hypothesis that modulation of angiogenesis and cardiac function by injecting small intestine extracellular matrix emulsion (EMU) into myocardium is associated with recruitment of c-kit cells, myofibroblasts, and macrophages after myocardial infarction.

Background Degradation of native extracellular matrix has been associated with adverse cardiac remodeling after infarction.

Methods Sixty-four rats were subjected to 45 min ischemia followed by 3, 7, 21, and 42 days of reperfusion, respectively. Saline or EMU (30 to 50 ml) was injected into the area at risk myocardium after reperfusion. Histological examination was performed by immunohistochemical staining, and cardiac function was analyzed using echocardiography.

Results The population of c-kit–positive cells in infarcted myocardium with the EMU injection increased significantly relative to the saline control at 7 days of reperfusion. Along with this change, alpha-smooth muscle actin expressing myofibroblasts and macrophages accumulated to a significant extent compared with the saline control. Increased vascular endothelial growth factor protein level and strong immunoreactivity of vascular endothelial growth factor expression were observed. Angiogenesis in the EMU area was significantly enhanced relative to the saline control, evidenced by increased density of α-smooth muscle actin positive vessels. Furthermore, echocardiography showed significant improvements in fractional shortening, ejection fraction, and stroke volume in the EMU group. The wall thickness of the infarcted middle anterior septum in the EMU group was significantly increased relative to the saline control.

Conclusions We show for the first time that injection of EMU into the infarcted myocardium increases neovascularization and preserves cardiac function, potentially mediated by enhanced recruitment of c-kit–positive cells, myofibroblasts, and macrophages. (J Am Coll Cardiol 2010;55:1250–61) © 2010 by the American College of Cardiology Foundation

The maladaptive ventricular remodeling initiated by myocardial infarction is characterized by progressive infarct expansion, wall thinning, and chamber dilation. During the early stage of myocardial infarction, the extracellular matrix (ECM) is degraded by matrix metalloproteinases leading to expansion of the infarcted zone. This is followed by a later phase of healing during which myofibroblasts proliferate and deposit collagen to form reparative fibrosis and scar tissue (1–4).

Over the years, much attention has been placed on tissue regenerative therapy with cell transplantation after myocardial infarction in an attempt to improve angiogenesis, replenish dead cardiomyocytes, and preserve cardiac function. However, it has not been consistently reported that invariant reconstitution of infarcted myocardium and blood vessels with cell transplantation is well integrated structurally and functionally in the ventricular wall (5–8). Recent studies have begun to provide promising alternatives to the typical cellular transplantation technique, using biomaterials to modulate cardiac repair. These biomaterials, including
fibrin glue (5), collagen (9), self-assembled nanopeptides (10), and alginate (11), can be directly injected into the infarcted myocardium to improve healing and remodeling. The mechanisms most likely involved include increased wall thickness and improved neovascularization (12).

Constructs of extracellular matrix emulsion (EMU) derived from acellular porcine small intestine submucosa have been shown to promote angiogenesis and the formation of cardiac tissue in patch studies of the right ventricle and atrial septum (13). The acellular EMU under study, which is an emulsion of the porcine small intestinal submucosa, primarily contains natural sources of glycoproteins (fibronectin and laminin) and glycosaminoglycans (heparan, hyaluronic acid, and chondroitin sulfate) in a complex of structures necessary for proper cell attachment and function. In addition, type I collagen with smaller amounts of type III, IV, V, and VI are also identified in this EMU.

However, we do not know whether this purified EMU promotes cardiac repair and improves cardiac function, and whether EMU has the capability to recruit endogenous cells responsible for protection when it is injected into the infarcted heart. Therefore, we tested the hypothesis that alleviation of maladaptive cardiac remodeling and improvement of cardiac function with intramyocardial injection of EMU are associated with mobilization of bone marrow resident progenitor cells (i.e., c-kit-positive cells), proliferation of myofibroblasts, and infiltration of macrophages.

Methods

Surgical preparation of animals. All animals received humane care in compliance with “The Guide for the Care and Use of Laboratory Animals” published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Male Sprague-Dawley rats weighing 400 to 450 g were anesthetized with an initial intraperitoneal injection of sodium pentobarbital (40 to 50 mg/kg) to induce anesthesia. The animals were intubated and mechanically ventilated with oxygen-enriched room air using a rodent respirator (Harvard Rodent Ventilator Model 683), the rate was adjusted to 30 to 40 breaths/min, and tidal volume was set to 1.1 to 1.3 ml/100 g body weight. Procedures on the first surgical day were performed under sterile conditions. The chest was opened by a left thoracotomy through the fourth intercostal space. After pericardiotomy, a 6-0 polypropylene ligature was placed under the left coronary artery (LCA) where it emerged from beneath the left atrium, and the ends of the tie were threaded through a small plastic tube (PE50) and mechanically stripped from the surrounding smooth muscle and serosal layers and from the tunica mucosa, often leaving intact the basilar layers. The remaining layer was predominately submucosa with the basement membrane. The remaining cellular elements were removed with 0.1% peracetic acid and rinsed in phosphate-buffered saline and distilled water. The sheets were cut to various lengths and freeze dried. They were then made into particulate by grinding and rehydrated with sterile saline to a suspension for injection.

Experimental protocol. The rats were randomly allocated to 1 of 4 groups. 1) Saline control group (n = 8 at each time point): saline (30 to 50 μl) was injected into the ischemic myocardium after 15 min of reperfusion. 2) Emulsion group (n = 8 at each time point): EMU (30 to 50 μl) was injected into the ischemic myocardium after 15 min of reperfusion. The rats in the control and EMU groups were subjected to 45 min LCA occlusion followed by 3, 7, 21, and 42 days of reperfusion, respectively. All injections were made through a 30G needle, and successful injection was visualized by the presence of EMU on the site of injection. 3) Sham group (n = 3 at each time point): the chest was opened and a ligature was placed under LCA without occlusion. 4) Sham plus EMU group (n = 3 at each time point): EMU was injected into the normal myocardium. Observational time points in the sham and sham plus EMU groups were the same as those in other experimental groups. After the injection was completed, the incisions were closed in layers. The chest and endotracheal tubes were removed when the animal was ventilator independent.

Cardiac function and wall thickness by echocardiography. Echocardiography was used to assess left ventricle (LV) systolic and diastolic function using a 2-dimensional guided M-mode ultrasound system (Acuson Sequoia, Siemens Medical Solutions, Mountain View, California) (15,16). Two-dimensional images from a short-axis view of the LV at the level of the papillary muscles were obtained using a 15 MHz linear transducer at a paper speed of 100 mm/s. Left ventricular percent fraction shortening and ejection fraction (EF) were calculated using the following equations: fraction shortening (%) was calculated as: \[
\text{FS} = \left(\frac{\text{LVDD} - \text{LVSD}}{\text{LVDD}}\right) \times 100
\]
and ejection fraction (%) was calculated as: \[
\text{EF} = \left(\frac{\text{EDV} - \text{ESV}}{\text{EDV}}\right) \times 100
\]
assess LV diastolic flow characteristics by calculating a ratio of early diastolic filling (E-wave) velocity and atrial filling (A-wave) velocity. The interventricular septum and posterior wall thickness were measured on the 2-dimensional images at the level of the papillary muscles, which was frozen at the end of diastole. The echocardiography was performed before opening the chest (baseline) and during the time course of the experiment in all groups. All measurements were averaged over 3 consecutive cardiac cycles.

**Histological preparation.** At the end of each experiment, the LCA was re-ligated and 1 ml of 20% Unisperse blue dye (Ciba-Geigy, Dover Township, New Jersey) was directly injected into the LV to outline the nonischemic area and risk myocardium. The animal was euthanized, and the heart was removed. The transverse slices including the nonischemic and ischemic zones were fixed in 10% phosphate-buffered formalin solution for 24 h, and embedded in paraffin for histological analysis. Cryosections (5-µm thick) were obtained using a Microtome (Leica RM2135, Houston, Texas). For Western blot analysis, the nonischemic and ischemic zones were immediately frozen in liquid nitrogen and kept at -70°C until use.

**Collagen deposition by Masson’s trichrome staining.** Masson’s trichrome is a 3-color staining protocol that stains collagen blue, nuclei black, and muscle fiber red. It has been previously validated through immunohistochemistry as an accurate technique for evaluating collagen deposition within muscle (Ancillary Pathways, Miami, Florida). Briefly, the tissue blocks were fixed in 10% buffered formalin and processed for paraffin embedding. Paraffin sections (5 µm) were hydrated with distilled water and stained with Masson’s trichrome, which determines the relative proportion of collagen deposition in the infarcted myocardium.

**Immunohistochemistry.** Immunohistochemical staining on tissue sections was performed as described previously (17). In brief, paraffin-embedded blocks were deparaffinized in xylene and dehydrated in graded ethanol. The prepared transverse paraffin sections were stained using the following antibodies: a polyclonal antibody against c-kit (C-19, Santa Cruz Biotechnology, Santa Cruz, California); a monoclonal anti-α-smooth muscle actin (SMA) (Clone 1A4, Sigma, St. Louis, Missouri); a polyclonal antibody against vascular endothelial growth factor (VEGF [147, Santa Cruz Biotechnology]); and a monoclonal antibody against macrophages (CD68, Millipore, Temecula, California). The slides were washed in phosphate-buffered saline, incubated with a biotinylated horse anti-rabbit immunoglobulin G (IgG) or anti–mouse IgG (Vector Laboratories, Burlingame, California), stained using the ABC-peroxidase kit or ABC-AR (alkaline phosphatase, Vector Laboratories), and then substrated with 3,3′-diaminobenzidine tetrahydrochloride or alkaline phosphatase substrate kit (Sigma). Quality of immunohistochemistry assay was controlled by either elimination of the primary antibody or incubation of the tissue with a nonimmune IgG. Data were analyzed in a blinded manner using computed-assisted morphometry (Image-Pro Plus 4.5, Media Cybernetics, Silver Spring, Maryland). Accumulation of α-SMA–expressing myofibroblasts and macrophages were calculated as mean number from 8 randomized high-powered fields on each slide. The density of arterioles defined as assessed from the average number of vessels that were positive for α-SMA. Expression of VEGF was similarly counted as mean density from 8 randomized high-powered fields on each slide and. Eight tissue blocks from each group were analyzed. All slides were evaluated at 200 times magnification.

**Western blotting analysis.** Western blotting was performed as described previously (17). In brief, freshly frozen nonischemic and ischemic tissue samples were homogenized in lysis buffer and protein concentration was measured by the DC Protein Assay (Bio-Rad, Hercules, California). The protein was then boiled and loaded onto gradient sodium dodecyl sulfate–polyacrylamide gel using Mini Protean II Dual Stab Cell (Bio-Rad). Membranes were subsequently exposed to a rabbit polyclonal antibody against stem cell factor (SCF [Santa Cruz Biotechnology]) and polyclonal antibody against VEGF (Santa Cruz Biotechnology). Bound antibody was detected by horseradish peroxidase conjugated anti-rabbit IgG. The membrane was incubated with chemiluminescence substrate and exposed to an x-ray film. The SCF was detected at bands of 45-31-kDa and VEGF at 21-kDa. The scanned images were imported into Adobe Photoshop, and the intensity of bands from the nonischemic and ischemic tissues was shown as arbitrary units.

**Statistical analysis.** Data were reported as the mean ± SE. A 1-way analysis of variance (SigmaStat, version 3.5, SPSS, Inc., Chicago, Illinois) followed by Student-Newman-Keul’s post-hoc test was used to analyze group differences in single point data. Echocardiographic data were analyzed by analysis of variance for repeated measures followed by post-hoc analysis with Student-Newman-Keul’s test for multiple comparisons. A p value of <0.05 was used to assign significant differences.

**Results**

A total of 76 rats were initially included in this study. Three rats in the control group and 2 rats in the EMU group died 2 days after the chest was closed because of a large area of infarction. Two rats in the control group and 3 rats in the EMU group died immediately during injection because of bleeding from an injured coronary artery. One rat in the control group and 1 rat in the EMU group were excluded because no evidence of infarct zone was found. The final 64 rats, namely, 8 rats in each observation period in both groups, were included for the histological examination and echocardiography. The mortality was comparable in the 2 groups, and successful EMU injection did not affect mortality during the 42 days of observation. An additional 24 rats were used in the sham group (12 rats) and sham plus EMU group (12 rats).
EMU localization and scar formation on the injection site over time of reperfusion. Injection of EMU into the ischemic/reperfused area was visible in the beating heart. The myocardium that received EMU swelled clearly at the injection site 3 days after injection (Fig. 1), indicating that the EMU does not escape into the left ventricular cavity. After 7 and 21 days of injection, EMU was still identified beneath the surface of the injection site, but was not clearly identified at 42 days. Masson’s trichrome staining revealed that the EMU engrafted homogenously within the injured myocardium, and aligned itself along the host myocardium without distorting ventricular wall geometry, as identified by demarcation of EMU in the myocardium (Fig. 2). In the control animals, the collagen deposition was clearly detected.
starting from 7 days of reperfusion. At 42 days, the collagen–rich myocardial scar extended through the entire ventricular wall and less viable myocardium was detected. However, the ischemic/reperfused myocardium appeared more organized and circumscribed in the EMU area. The ventricular wall thickness at the site of EMU zone was well preserved. At 42 days of injection, a smaller amount of fibrous tissue was identified within the EMU zone, suggesting tissue regeneration. No collagen deposition was detected in the nonischemic zone in both control and EMU groups as well as in the sham and sham plus EMU groups (data not shown).

**Expression of SCF in myocardium.** SCF is expressed in the larger 45-kDa form and subsequently cleaved to produce a smaller and soluble 31-kDa form when it is activated. SCF in the 31-kDa form is the ligand for the transmembrane tyrosine kinase receptor proto-oncogene c-kit. As shown in Figure 3, the SCF in 45-kDa form was detected in the nonischemic zone in both control and EMU groups. Three days of reperfusion in the control group did not alter SCF expression of 45-kDa form, but its level was significantly increased after 7 days. No significant cleavage of the larger 45-kDa form was found relative to the nonischemic zone in the control animals over the time of reperfusion. However, EMU injection significantly increased SCF expression in both 45- and 31-kDa forms during the entire period of reperfusion compared with the control group. No statistically significant difference in levels of SCF in larger and smaller forms was detected in the nonischemic zone in both control and EMU groups as well as in the sham and sham plus EMU groups (data not shown).

**Recruitment of c-kit–positive cells in myocardium.** No c-kit–positive cells were detected in the nonischemic myocardium throughout reperfusion in the control and EMU groups as well as in the sham and sham plus EMU groups. However, c-kit–positive cells were detected in the myocardium at 3 days in the control animals, with a significant increase in the number of c-kit–positive cells at 7 days of reperfusion (Fig. 4). Relative to the control animals, the c-kit–positive cells were significantly increased in the EMU injection site, with a peak in recruitment of c-kit–positive cells at 7 days. At 21 and 42 days of reperfusion, c-kit–positive cells in the EMU group declined, but were still statistically greater than those in the control group.

![Figure 3: Expression of SCF in Ischemic/Reperfused Myocardium](image-url)

Stem cell factor (SCF) in larger 45-kDa form was detected in the nonischemic zone (N) in both the control group and extracellular matrix emulsion (EMU) group. However, injection of EMU (solid bars) significantly increased SCF levels in all forms relative to the control (shaded bars) during reperfusion. Ponceau S staining (Po-S) shows equal amount of protein loading. Values are mean ± SEM (n = 8). *p < 0.05 versus the nonischemic zone (normal); †p < 0.05 EMU versus control.
Accumulation of \( \alpha \)-SMA–expressing myofibroblasts and macrophages in myocardium. As shown in Figure 5A, \( \alpha \)-SMA–expressing myofibroblasts were detected in the nonischemic myocardium in the control and EMU groups. Relative to the number in the nonischemic myocardium, \( \alpha \)-SMA–expressing myofibroblasts were significantly increased after 3 days of reperfusion in the control group, but reduced during subsequent reperfusion periods. However, EMU injection further augmented the number of \( \alpha \)-SMA–expressing myofibroblasts in the myocardium relative to each time point of reperfusion compared with the control group, with a peak at 7 days.

Macrophages were detected in the nonischemic myocardium in both the control group and the EMU group, as shown in Figure 5B. Ischemia/reperfusion caused a significant increase in the macrophage accumulation in myocardium relative to the nonischemic zone, with a peak at 3 days of reperfusion. There was a trend toward an increase in macrophage accumulation in the myocardium compared with the control group, with a peak at 7 days.

There was a significant linear relationship in the number of accumulated macrophages and \( \alpha \)-SMA–expressing myofibroblasts at 7 days of reperfusion (y = 27 + 0.153\*X, r = 0.814, p < 0.05), suggesting that the migrated macrophages are potentially involved in recruitment of \( \alpha \)-SMA–expressing myofibroblasts during reperfusion.

The \( \alpha \)-SMA–expressing myofibroblasts and macrophages were not detected in myocardium in the sham and sham plus EMU groups (data not shown). There were only minor signs of foreign-body reaction with EMU injection as hematoxylin and eosin staining revealed comparable level in neutrophil accumulation within the infarcted zone in both control and EMU groups.

VEGF expression and angiogenesis in infarcted myocardium. The VEGF protein was detected in the sham group and the sham plus EMU group, but no statistical difference was found relative to the nonischemic myocardium among these groups (data not shown). Ischemia/reperfusion caused a significant increase in the level of VEGF protein at 3 and 7 days of reperfusion. However, its level was subsequently reduced at 21 and 42 days (Fig. 6A). Compared with the control animals, EMU injection significantly increased the level of VEGF protein with a peak at 7 days. Starting from 21 days, VEGF levels in the EMU group were reduced,
but were still significantly higher than those in the control group. Consistent with these results, VEGF expression was also affected by EMU injection, as shown in Figure 6B. Ischemia/reperfusion increased intensity of immunohistochemical VEGF staining in the control group at 3 days, but its expression was reduced during the following time periods of reperfusion. However, there was a significant increase in the expression of VEGF in the myocardium throughout the entire period of reperfusion in the EMU group.

Enhancement in the level and expression of VEGF by injecting EMU was accompanied by an increase in newly formed microvessels within the EMU zone. As shown in Figure 7, ischemia/reperfusion significantly increased the density of α-SMA–positive microvessels during 3 and 7 days of reperfusion relative to the nonischemic myocardium, suggesting that the stress initiates a compensating response to ischemia/reperfusion injury. However, the mean density of α-SMA–positive microvessels was subsequently reduced after 21 days in the control animals. The EMU injection significantly increased the density of α-SMA–positive microvessels in the infarcted zone relative to each time point observed. Values are mean ± SEM (n = 8). *p < 0.05 versus nonischemic zone (normal); †p < 0.05 EMU (solid bars) versus control (shaded bars). HPF = high-powered field. Magnification ×200.

Echocardiographic evaluation of cardiac function and wall thickness. The results from echocardiographic evaluation are summarized in Table 1. No significant statistical difference was found in all echocardiographic parameters measured in the sham and sham plus EMU groups, so data from these 2 groups were averaged. During 42 days of reperfusion in the control group, LVSD, LVDD, EDV, and ESV were significantly higher compared with the baseline values. In addition, the stroke volume was significantly lower and indexes of systolic function, namely, fraction shortening and EF were significantly reduced, suggesting contractile dysfunction (Fig. 8). An example of pulsed-wave Doppler recordings of mitral inflow is shown in Figure 9A. Ischemia/reperfusion increased both early diastolic filling (E-wave) velocity and atrial filling (A-wave) velocity, and therefore resulted in a significant decrease in the ratio of E-wave to A-wave velocity (E/A) relative to baseline values in the control animals. The EMU injection significantly improved changes in systolic and diastolic functions measured in all time points relative to those in the control group (Table 1, Figs. 8 and 9A). However, the improvements in systolic and diastolic functions secondary to EMU injection partially declined relative to the baseline values during the periods of 42 days of reperfusion, suggesting lack of full protection by a single EMU injection.
The wall thickness of the infarcted middle anterior and posterior septum was measured from echocardiographic images. As shown in Figure 9B, ischemia/reperfusion caused significant wall thinning at 42 days, but this change was preserved with EMU injection. The wall thickness of infarcted middle anterior septum in the EMU area was significantly increased relative to that in the control animals (1.8 ± 0.02 mm vs. 0.9 ± 0.01 mm, p < 0.01). However, the wall thinning was still identified in infarcted middle anterior septum that did not receive EMU injection, as indicated by an arrowhead in Figure 9B.

The wall thickness of the infarcted middle anterior and posterior septum was measured from echocardiographic images. As shown in Figure 9B, ischemia/reperfusion caused significant wall thinning at 42 days, but this change was preserved with EMU injection. The wall thickness of infarcted middle anterior septum in the EMU area was significantly increased relative to that in the control animals (1.8 ± 0.02 mm vs. 0.9 ± 0.01 mm, p < 0.01). However, the wall thinning was still identified in infarcted middle anterior septum that did not receive EMU injection, as indicated by an arrowhead in Figure 9B.
Sham EF SV revealed that the injection of EMU into the infarcted myocardium significantly increases expression of stem cell factor and recruits bone marrow–derived c-kit–positive cells. Consistent with enhanced angiogenesis, EMU injection promoted α-SMA–expressing myofibroblast infiltration and macrophage accumulation. Accordingly, EMU injection increased ventricular wall thickness and the improved cardiac functions. These data suggest that the favorable effects of this xenogeneic EMU on cardiac remodeling may relate to an improvement of microenvironment during tissue repair after infarction.

Table 1  
**Echocardiographic Data**

<table>
<thead>
<tr>
<th>Index</th>
<th>Control Group</th>
<th>Emulsion Group</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Base 3 7 21 42</td>
<td>Base 3 7 21 42</td>
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<tr>
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<td>1.9 ± 0.6</td>
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<td>LVSS, mm</td>
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<tr>
<td>Post-sep, mm</td>
<td>2.1 ± 0.1</td>
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Values are mean ± SEM. *p < 0.05 versus baseline values; †p < 0.05 versus control group.

Discussion

After myocardial infarction, ECM turnover is one of the most important factors in retaining tissue integrity and preventing maladaptive remodeling. A normal ECM provides the appropriate cellular microenvironment for cell migration, growth, and differentiation, and also serves as a storage depot for growth factors, hormones, and cytokines responsible for cellular signaling functions (1,2). This study revealed that the injection of EMU into the infarcted myocardium significantly increases expression of stem cell factor and recruits bone marrow–derived c-kit–positive cells. Consistent with enhanced angiogenesis, EMU injection promoted α-SMA–expressing myofibroblast infiltration and macrophage accumulation. Accordingly, EMU injection increased ventricular wall thickness and the improved cardiac functions. These data suggest that the favorable effects of this xenogeneic EMU on cardiac remodeling may relate to an improvement of microenvironment during tissue repair after infarction.

**Figure 8  Cardiac Systolic Function During the Course of the Experiment**

Cardiac function was detected by echocardiography using a 2-dimensional guided M-mode ultrasound system in the control group (solid squares), extracellular matrix emulsion (EMU) group (open circles), and sham group (solid triangles). Ischemia/reperfusion caused a significant reduction in contractile function evidenced by reduced fraction shortening (FS), ejection fraction (EF), and stroke volume (SV). Relative to each time point in the control group, EMU injection significantly preserved cardiac systolic function during reperfusion. No changes in cardiac systolic and diastolic function were detected in the sham group. Values are mean ± SEM (n = 8). *p < 0.05 versus respective baseline values in the control group and EMU group; †p < 0.05 versus control group.
Stem cell factor is a pleiotropic cytokine that has 2 alternatively spliced forms: the membrane-bound larger 45-kDa form and the smaller soluble 31-kDa form. The larger SCF form is primarily expressed in fibroblasts and can be cleaved to produce soluble 31-kDa form by multiple factors, such as granulocyte colony-stimulating factor, stromal cell-derived factor-1, or VEGF (18). With respect to a role of SCF in cardiac remodeling, it has been reported that SCF enhances endogenous cardiac repair working through recruitment of the bone marrow–derived c-kit progenitor cells and other differentiated precursor cells (18,19). Recent studies have provided direct evidence showing that SCF critically affects cardiac healing after myocardial infarction by modulating differentiation of myofibroblasts from c-kit cells and stimulating angiogenesis through VEGF released from myofibroblasts (19). In the current study, we found that the soluble SCF level in the control group was significantly detected only at 7 days of reperfusion, which corresponded to a significant accumulation of c-kit–positive cells, and then returned back to the baseline level at 21 and 42 days (18,20). However, EMU injection augmented SCF levels at 3 days of reperfusion, and its increased level was constantly maintained throughout 42 days. These data were consistent with the time course in increased number of c-kit–positive cells in the EMU region, suggesting a role of SCF/c-kit system in EMU-modulated cardiac repair. Since c-kit–positive cells were also detected in the infarcted myocardium of the control group, we cannot exclude the possibility that resident c-kit–positive cells were accumulated in the myocardium after infarction. However, a significant increase in the number of c-kit–positive cells with EMU injection suggests that these cells are largely derived from the bone marrow (18).

Myofibroblast, a specialized phenotype of cell derived from fibroblast that expresses α-SMA, has been described as a contractile cell for granulation repair tissue. In response to myocardial infarction, accumulation of myofibroblasts in the infarcted myocardium may contribute to scar tissue stabilization by indirectly regulating secretion of angiogenic factors for blood vessel construction and directly depositing collagen for scar tissue formation (21–26). In the current study, although a significant number of α-SMA–expressing...
myofibroblasts was detected after 3 days post-infarction, the accumulation of myofibroblasts declined between 7 and 42 days in the control animals, which is a period responsible for maintaining structural integrity and wound closure of healing scars (21,22). The origin of indigenous myocardial myofibroblasts is unresolved, but recent studies have demonstrated that formation of myofibroblast-rich repair tissue was significantly inhibited when the bone marrow–derived c-kit cells are mutated. In these studies, replacement of the mutant bone marrow with c-kit cells isolated from the wild-type animals rescued the myofibroblast proliferation, suggesting a role of c-kit cells in transdifferentiation of myofibroblast phenotype (18). The current study raises the possibility that injection of EMU preserves natural sources of ECM, protects native ECM from degradation, and therefore provides a cellular microenvironment for c-kit cell migration and differentiation. Compared with the control animals, the number of infiltrated α-SMA–expressing myofibroblasts in EMU region was significantly increased at 7 and 21 days after reperfusion, consistent with an increase in accumulation of c-kit–positive cells during these time periods. These data suggest that mobilization of c-kit–positive cells from the bone marrow facilitated by EMU may increase the myofibroblast differentiation, enhance angiogenesis, and thereby drive efficient endogenous cardiac repair. In this regard, the mobilization of the bone marrow–derived cells to the small intestinal submucosa has been previously demonstrated to participate in the tissue remodeling process in other applications (27).

Infiltrating macrophages into the damaged tissue has been associated with myofibroblast accumulation and angiogenesis. A recent study has reported that macrophage depletion with intravenous injection of clodronate-containing liposomes reduces infiltration of myofibroblasts and intensity of neovascularization after myocardial injury. Along with these changes, the wound healing was impaired and the mortality was increased (28). In addition, direct injection of human macrophages into the infarcted myocardium in rat promoted vascularization, enhanced myofibroblast accumulation, and improved cardiac function (29). In the current study, we found that injection of EMU significantly increased macrophage accumulation when compared with the control animals during 42 days of reperfusion, suggesting a role for macrophage accumulation in cardiac repair. A linear relationship between macrophages and α-SMA–positive cells accumulated at 7 days of reperfusion may indicate that migration of macrophages is potentially responsible for myofibroblast accumulation after EMU injection.

Formation of microvessels in the infarcted myocardium is crucial during the healing process. Although the mechanisms responsible for the neovascularization after myocardial infarction have not been fully elucidated, an increase in populations of α-SMA–expressing myofibroblasts and macrophages in the infarcted myocardium has been associated with enhanced angiogenesis after myocardial infarction (21,28). Myofibroblasts and macrophages secrete large amount of VEGF, a critical angiogenic factor for inducing neovascularization in granulation repair tissue, when these cells are activated after myocardial infarction (21,28). In the current study, we demonstrated the dynamic alterations in VEGF expression and microvessel density during maturation of the healing myocardial scar, and assumed that these changes modulated by EMU are associated with increased accumulation of α-SMA–expressing myofibroblasts and macrophages. These data signified an important relationship among neovascularization, myofibroblasts and macrophages in EMU-enhanced cardiac angiogenesis.

Intramyocardial injection of several biomaterials into the heart have shown the beneficial effects on tissue remodeling after myocardial infarction, most likely due to the mechanical support and the wall tension reduction (5,9,12). In the current study, we found that EMU injection significantly improves cardiac systolic and diastolic function for as long as 42 days of reperfusion when compared with the saline control group. These data were consistent with a significant increase in the number of c-kit–positive cells, accumulation of α-SMA–expressing myofibroblasts and macrophages, as well as higher levels of newly constructed blood vessels. In addition to these biological effects, the EMU may also prevent maladaptive cardiac remodeling by providing structural support to the ventricular wall where they form 3-dimensional matrices upon injection, conform to the geometry of the ventricular space, and increase ventricular wall thickness. Therefore, the capability of EMU to deform with dynamically improved myocardial microenvironment and to align with the injured myocardium may provide better incorporation in cell recruitment and cardiac recovery.

This is not a study to show the “cause-effect” relationships among cell migration, cell proliferation, and tissue angiogenesis during cardiac healing with EMU injection. We selected recruitment of several different cell types, expression of VEGF, and formation of blood vessels as markers, which have been previously demonstrated to be beneficial in tissue regeneration (11,12), to show protective effects of EMU injection after myocardial infarction. However, there is a limitation in this rat model of ischemia and reperfusion with EMU injection. The thickness of ventricular anterior wall in rat is <2 mm; it is very difficult to apply for multiple injections to fully cover the entire area at risk myocardium in the same heart. Because we still see the decline in cardiac function over time of reperfusion (Figs. 8 and 9), future work in a larger animal model is warranted to demonstrate whether multiple EMU injections to a single heart could provide the maximal protection. In addition, more work is needed to determine what the optimal elements of EMU in modulating remodeling are, and how EMU protects the heart through cell migration and differentiation.
Conclusions
This is the first histological and functional study to demonstrate the beneficial effects of EMU injection on mal-adaptive cardiac remodeling. Relative to other materials injected into the heart after myocardial infarction (S,9–11), we assume that this natural EMU is able to provide the host a proper matrix microenvironment for cell survival and retention in the myocardium by its complex structures, which may be favorable not only in providing mechanical support but also in improving cardiac function. These data provide direct experimental evidence to show whether adverse cardiac remodeling could be modified by supplying ECM after myocardial infarction.

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Reprint requests and correspondence: Dr. Zhi-Qing Zhao, Department of Basic Biomedical Sciences, Mercer University School of Medicine, 4700 Waters Avenue, Savannah, Georgia 31404. E-mail: zhao_z@mercer.edu.

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