c-Kit Dysfunction Impairs Myocardial Healing After Infarction

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Background—We hypothesized that c-kit receptor function in the bone marrow is important for facilitating healing, leading to efficient cardiac repair after myocardial infarction (MI).

Methods and Results—We used Kit\(^{W/KitW}\) c-kit mutant mice and their wild-type littermates to assess the importance of c-kit function in cardiac remodeling after coronary ligation. We found that mutant mice developed 1.6-fold greater ventricular dilation (P=0.008) attributable to a 1.3-fold greater infarct expansion by day 14 after MI (P=0.01). The number of proliferating smooth muscle \(\alpha\)-actin expressing cells was 1.8-fold lower in mutant mice at day 3 (P<0.01), resulting in a 1.6 to 1.8-fold reduction in total regional nonvascular smooth muscle \(\alpha\)-actin expressing cells by both microscopy and flow cytometry (P<0.001 for both). This decrease was accompanied by a 1.4-fold reduction in the number of CD31 expressing blood vessels (P<0.05). Prior transplantation of wild-type bone marrow cells into mutant mice rescued the efficient establishment of vessel-rich repair tissue by inducing a 1.5-fold increase in nonvascular smooth muscle \(\alpha\)-actin expressing cells and CD31 expressing blood vessels (P<0.05 for both). The increased recruitment of cells into the infarct region in the chimeric mice was associated with reduced infarct expansion (P<0.03) compared to wild-type levels.

Conclusions—Bone marrow c-kit function critically impacts the myofibroblast repair response in infarcted hearts. Interventions that increase the infiltration of c-kit\(^{+}\) cells to the infarcted heart may potentiate this endogenous repair response, prevent infarct expansion, and improve the recovery of cardiac function after MI. (Circulation. 2007; 116[suppl I]:I-77–I-82.)

Key Words: c-kit \[\mathbb{\bullet}\] myofibroblast \[\mathbb{\bullet}\] myocardial infarction \[\mathbb{\bullet}\] cardiac remodeling

Cardiac repair post-MI entails a robust inflammatory response that removes necrotic tissue and precedes the appearance of repair tissue, rich in myofibroblasts and microvessels. Echocardiographic data from patients after an anterior MI demonstrated that the endocardial length of the infarcted segment stabilizes and may decrease over the first year after infarction whereas the noninfarcted segment length increases.\(^1\) A rapid increase in the number of smooth muscle \(\alpha\)-actin (SMA) expressing myofibroblasts\(^2\) likely accounts for infarct stabilization and prevention of ongoing infarct expansion, as in other models of wound healing.

Although 90% of cardiac mass is contributed by cardiomyocytes, these cells only comprise 25% to 33% of total cell number. The remaining cells are nonmyocytes, the majority of which are fibroblasts and endothelial cells.\(^3\) After MI, fibroblasts proliferate and express SMA, a phenotype that characterizes the myofibroblasts and requires TGF-\(\beta\).\(^2,4\) Myofibroblasts then undergo progressive apoptosis in the wound contracture phase.\(^5\) Transient inhibition of apoptosis after an MI improves late remodeling and survival by temporarily increasing the number of myofibroblasts in the infarcted region.\(^6,7\)

We recently reported that in the absence of c-kit\(^{+}\) cell mobilization from the bone marrow, infarcted hearts undergo rapid ventricular dilation and cardiac failure.\(^8\) Cells that express the c-kit tyrosine kinase receptor include hematopoietic stem cells, mast cells (MCs), endothelial progenitor cells (EPCs), and the putative resident cardiac stem cells.\(^9\) We found that a loss of bone marrow–derived c-kit\(^{+}\) EPC mobilization results in diminished endothelial mitogenesis and impaired infarct borderzone angiogenesis, demonstrating the importance of the c-kit/SCF pathway in cardiac repair. Because myofibroblast accumulation and angiogenesis are interlocking events important in granulation tissue formation,\(^10\) we used experiments to further understand the impact of c-kit dysfunction on myofibroblast proliferation and accumulation.

Methods

Experimental Model of Myocardial Infarction

The Animal Care Committee of the Toronto General Research Institute approved all procedures performed on animals. All experiments were performed according to the Guide to the Care and Use...
of Experimental Animals of the Canadian Council on Animal Care, and the Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, revised 1985). Eight to 12-week-old female wild-type Kit+/+ (n=51) and mutant Kit+/Kit−/− (n=54) mice were used, and a permanent left anterior descending artery ligation was performed as described.8

Bone Marrow Reconstitution
We injected 2.5×10⁷ fresh bone marrow cells from Kit+/+ (n=5) donors prepared in an aseptic fashion into the tail veins of irradiated (9.5 Gy) Kit+/Kit−/− mice (n=10). After 4 to 6 weeks, reconstitution was assessed in the bone marrow transplanted (BMT) group (Kit−/+→Kit+/Kit−/−) by polymerase chain reaction as described previously.5

Cardiac Function
Echocardiography was performed in 8 animals per group (8 Kit−/+ mice, 8 Kit+/Kit−/− mice) at day 7 after MI, and 6 animals per group (6 Kit−/+ mice, 6 Kit+/Kit−/− mice) at day 14. BMT mice (n=5) were assessed at day 14.

Animals were sedated with 2% isoflurane, and 2-D images were obtained using a 15 MHz linear array probe and a Sequoia echocardiography system (Siemens). Cardiac cycles were recorded. Offline measurements of scar length were assessed in a blinded fashion from short axis views at the mid-papillary level of the left ventricle (LV), and were expressed as the percentage of the entire LV circumference.

Heart Morphological Measurements
Fourteen mice (7 Kit−/+ mice, 7 Kit+/Kit−/− mice) were euthanized via anesthetic overdose at 14 days after MI. Hearts were perfused with KCl supplemented with heparin. Formalin (10%) was injected into the aorta and the LV distended. Hearts were immersion fixed in formalin for 48 hours, sectioned transversely (1 mm thickness), and photographed. The normal myocardium and myocardial scar were quantified using computerized planimetry (Image J, NIH Software). The total LV volume is presented as the sum of the luminal areas. From this tissue, paraffin-embedded toluidine blue stained sections were generated.8

Immunofluorescent Staining and Confocal Microscopy
Hearts from 59 mice (27 Kit+/+, 27 Kit+/Kit−/− and 5 Kit−/+→Kit+/ Kit−/−; bone marrow chimeras) were harvested and sliced 3 mm below the ligature at day 0, and days 1, 3, 7, and 21 postinfarction. Sections incubated with SMA-Cy3 (1:300, Sigma) alone, or with SMA-Cy3 and Ki67 (1:100, Dako) were post-fixed in methanol:acetone (1:1) and permeabilized with 0.5% triton X-100. In separate experiments, SMA-Cy3 was incubated with anti-mouse CD31 (1:400, Invitrogen) after postfixation in acetone. Permeabilization was achieved after incubation with anti-CD31 using 0.05% triton X-100 washes. Appropriate secondary antibodies were used and nuclei identified with Hoechst 33342. Postfixed and secondary-only incubated sections were used to set detector gains and voltage before scanning. Using an Olympus Fluoview 2000 laser scanning confocal microscope, 5 random fields of view were acquired from the remote, borderzone, and infarct regions.

Image Analysis
Using Image J software, SMA’ pixel area was measured and expressed as percentage of total possible pixels. In CD31/SMA-stained sections, SMA’ coated blood vessels (≥30 µm) were identified and digitally removed. The remaining SMA’ pixels were quantified to yield the % of nonvascular SMA’ pixels. Ki67’ nuclei surrounded by SMA’ pixels were counted and expressed as the number of SMA’/Ki67’ cells per mm².

Flow Cytometry
Cell preparations were established from heart digests (14 Kit−/+ mice and 14 Kit+/Kit−/− mice) and stained as described previously.8 Cy3-anti-SMA and PE-anti-FcαR1a antibodies were used. For Cy3-anti-SMA staining, cells were fixed and permeabilized for intracellular staining using the Cytofix/Cytoperm kit (BD Biosciences). Samples were analyzed using an FC500 flow cytometer (Becton Dickinson). Unstained cells were used to set parameters before cytometric analysis.

Statistics
Data are presented as mean±SEM. Time-course and multigroup analyses were carried out using analysis of variance (ANOVA). Bonferroni and Newman-Keuls post test analysis was used to detect differences between groups. A t test with Welch correction was used to compare groups at a single time point. *P<0.05 was considered statistically significant.

Statement of Responsibility
The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results
LV Volume and Scar Length Increase in Kit+/Kit−/− Mice as Early as Day 14 Post-MI
Kit+/Kit−/− and Kit−/+ mouse heart morphometry was assessed 14 days after MI (Figure 1A). LV volume (Figure 1C) increased significantly in the Kit−/+/Kit−/− mice compared with wild-type (P<0.01). Concomitantly, infarcted segment thickness was reduced (P<0.023) and infarct area was increased.
Transient accumulation of SMA immunofluorescently stained sections. We found rapid but
1.6-fold reduction at day 7 (Figure 1B, P < 0.01). At day 9 (n = 3 per strain), 3, and
21 (n = 5 per strain) no differences were detected. J and K, Flow cytometric analysis
confirmed the decreased SMA² cells at 7 days after MI. Overlays of representative
histograms demonstrate a decreased area under the curve in mutants compared with
wild-type mice (J). Quantification of the flow cytometric data (K) confirms the histomor-
phometric analysis with an increase in SMA² cells in wild-type (n = 7) over mutants
(n = 7, * P < 0.001).

**Kit²/²** Mice Mount a Diminished SMA² Cell
Response Post-MI

We next evaluated the formation of SMA² rich tissue using immunofluorescently stained sections. We found rapid but
transient accumulation of SMA² tissue in infarcted myocardium that peaked 7 days post-MI (Figure 2A through 2H).
This phenomenon was blunted in mutant mice, with a 1.6-fold reduction at day 7 (P < 0.001). No differences were
found at other time points. To confirm this finding, we used flow cytometric analysis to analyze the number of SMA²
cells released by collagenase digestion from whole hearts. We found that 7 days post-MI, the mutant mice had a 1.8-fold
reduction in total SMA² signal (Figure 2J and 2K, P < 0.001).

**Myofibroblast Proliferation Is Diminished Post-MI in**
**Kit²/²** **Mice**

Previously, we found a reduced number of Ki67² nuclei in
Kit²/² post-MI. 8 In this study we determined whether a
contributing subset of cells were spindled-shaped and SMA²,
that identified as myofibroblasts (Figure 3). SMA²/Ki67²
dual positive cells were quantified at days 1, 3, and 7 post-MI.
A 1.8-fold reduction in the number of SMA²/Ki67² cells was
found in the borderzone of Kit²/² mice at day 3 only
(Figure 3; P < 0.01). Surprisingly, no difference was detected
in the infarcted region. Because myofibroblasts migrate into
the infarct region from the borderzone, 11 we suspect the
reduction in proliferating cells in the borderzone at day 3 is
likely responsible for the reduction of myofibroblasts in the
infarcted region at day 7.

**Kit²/²** **Mice Lack Cardiac Mast Cells**

Mast cells (MCs) are important partners in the myofibroblast
response. 12 Kit²/² mice lack tissue MCs, but possess bone
marrow derived MCs. 13 Using flow cytometry (Figure 4A),
we found that the number of cardiac FcεRIα² cells was
2.7-fold greater in wild-type mice (n = 3 per strain, P < 0.05). Because FcεRIα² cells include basophils and macrophages, 14 we also assessed tolu-
idine blue stained sections for the presence of metachromatic
MCs at days 0 and 3 postinfarction (Figure 4C). Kit²/² mice

![Figure 2. SMA² cell accumulation was reduced in mutant mice compared with wild-type mice. A-H, SMA accumulation (red fluorescence) in the infarcted segments over time in both wild-type and mutant mice. At day 7, the SMA² area was diminished in mutants compared with wild-type mice. I, Quantification of the images confirmed this difference at day 7 (n = 5 per strain, * P < 0.001). At day 9 (n = 3 per strain), 3, and 21 (n = 5 per strain) no differences were detected. J and K, Flow cytometric analysis confirmed the decreased SMA² cells at 7 days after MI. Overlays of representative histograms demonstrate a decreased area under the curve in mutants compared with wild-type mice (J). Quantification of the flow cytometric data (K) confirms the histomorphometric analysis with an increase in SMA² cells in wild-type (n = 7) over mutants (n = 7, * P < 0.001).](image)

![Figure 3. SMA² cell proliferation was reduced in the borderzone region at day 3 in the mutant but not the wild-type mice. A and B, Representative micrographs of borderzone images from wild-type (A) and mutant (B) mice. Digitally zoomed inset images (white rectangles) demonstrate the SMA²/Ki67² cells (arrows). C and D, The number of SMA²/Ki67² cells per mm² of borderzone (C) was quantified at day 1 (n = 3 per strain), 3, and 7 (n = 5 per strain per timepoint) and was increased in wild-type over mutants at day 3 post-MI (* P < 0.01). No difference was found in the scar region (D).](image)
Bone Marrow Reconstitution Rescues the Blunted Myofibroblast Response in the KitW/KitW-v Mice

We next assessed the myofibroblast response in the hearts of the mutant mice after BMT with wild-type bone marrow cells (BMT, Kit+/+ → KitW/KitW-v). Quantification of nonvascular SMA pixels in double-stained (anti-SMA/anti-CD31) tissue sections showed a rescue of the myofibroblast response (Figure 5G, P=0.019) in infarcted hearts of BMT mice compared with KitW/KitW-v mice. There was also a concomitant improvement in the blood vessel density estimated by CD31 structure quantification (Figure 5I, P=0.034) and an increasing trend in the percentage of vascular SMA pixels (Figure 5H, P=0.058). To rule out the possibility of irradiation predisposing BMT mice to an increased number of myofibroblasts, areas remote to the infarct were also surveyed (Figure 5D through 5F) and no myofibroblasts were observed.

To determine whether the increased myofibroblast accumulation in BMT mice was associated with improved scar dynamics, we again assessed scar length by echocardiography. We found a reduction in scar length in BMT mice back to Kit+/+ levels (Figure 5J, P=0.03).

Discussion

Angiogenesis and granulation tissue formation are interlocking events in infarct repair.10 Key cellular mediators in this response are myofibroblasts, which are known to produce VEGF15 and endothelin,16 and have been suggested to contribute to blood vessel formation.17,18 Myofibroblast-mediated scar contracture is required to avoid the thinning and dilatation that results in congestive heart failure.3 Increased infarct zone myofibroblast accumulation has been associated with favorable cardiac remodeling and improved cardiac function.19–21

We previously demonstrated that c-kit dysfunction is associated with reduced angiogenesis after MI.8 In this report, we evaluated whether a reduction in c-kit cells would impact myofibroblast accumulation and contribute to cardiac dilatation and dysfunction. In KitW/KitW-v mice, we found that proliferating myofibroblasts were reduced 3 days after MI, and that myofibroblast accumulation was diminished 7 days after MI. In addition, BMT with healthy c-kit cells restored the cardiac myofibroblast response, reduced scar length, and prevented cardiac dilatation and dysfunction 14 days after the infarct.

Our finding of diminished SMA myofibroblasts is supported by previous findings that KitW/KitW-v mice are protected from diastolic failure induced by pressure-overload–mediated cardiac fibrosis.12 Because cardiac fibroblasts and myofibroblasts are the primary mediators of cardiac fibrosis in long-term pressure overload, blunted myofibroblast accumulation may explain the protection of KitW/KitW-v mice in that model, and the precipitous decline in cardiac function after MI in this study. Diminished myofibroblast accumulation may have prevented scar stabilization in the KitW/KitW-v mice, and may explain their limited survival. The increased mortality that we previously observed in mutant mice between 7 to 20 days post-MI corresponded to the period of scar stabilization required to maintain structural integrity of the injured myocardium in wild-type mice.2

Potential mechanisms responsible for the decreased accumulation of myofibroblasts include the elaboration of paracrine mediators by c-kit+ cells, which stimulate not only angiogenesis but also myofibroblast accumulation. Because
we have previously observed decreased migration, infiltration, and proliferation of c-kit+EPCs after MI, the lack of c-kit+ cells in the postinfarct heart could reduce both indirect and direct myofibroblast accumulation. Indirect modulation of the myofibroblast population is supported by Dimmeler’s group, who found that EPCs, which express c-kit, produce mRNA of factors known to impact myofibroblasts, including FGFα, SDF-1, PDGF, and TGF-β.22 Other cells of angiogenic potential that could have contributed to the SMA+ population found in our study include stimulated pericytes that may become myofibroblasts and contribute to infarct repair.23

Another indirect mechanism for decreased myofibroblast accumulation could be mediated through MCs, which are diminished in Kitw/Kitw-vv mice. Decreased myofibroblast infiltration and proliferation around the infarct region could be associated with decreased MCs in the mutant mice. However, consistent with other observations,24 we also found very few MCs in the wild-type myocardium after MI. Thus, it is unlikely that MCs played a significant role in our model.

We have previously shown that c-kit+ cells directly give rise to SMA+ cells in vitro and in vivo after femoral artery injury in mice.25 SMA+ expressing cells were also observed when EPCs grown on a scaffold were stimulated with TGF-β, in an effort to reconstruct a cardiac valve.26 Therefore, the c-kit+ cell population may act by directly differentiating into SMA+ cells. Collectively, these findings may have clinical relevance for patients who have experienced a heart attack. Such patients have a diminished marrow regenerative capacity27 similar to that of our mutant mice. Myofibroblast accumulation and infarct scar stabilization may be achieved in these patients either by implanting stem cell factor overexpressing cells into the infarct region,28 or by rejuvenating dysfunctional c-kit progenitor cells via bone marrow transplantation.2

**Figure 5.** Bone marrow transplantation (BMT) restored the accumulation of myofibroblasts in mutant mice. A-F, Representative micrographs shows double stained tissue (CD31, green and SMA, red fluorescence), that allowed for the removal of SMA+ coated blood vessels and microvascular endothelial cells. The pixel area of SMA+ cells was greater in the infarct region in the BMT (Kitw/+ → Kitw+/Kitw+v C) compared with mutant mice (Kitw+/Kitw-vv B), but not different than wild-type. The remote areas (D-F) demonstrated that irradiation did not increase SMA+ myofibroblasts, suggesting that the increase in the infarcted segment was attributable to restored bone marrow c-kit+ cell function. G, Quantification demonstrated that the nonvascular SMA+ pixel area was reduced in the mutant mice (n = 6; P = 0.003) and restored in BMT mice (n = 5; P = 0.019) which was not different than wild-type (n = 6; P = 0.25) mice. H, The percentage of SMA+ pixels attributed to vascular structures was decreased in the mutant mice (compared with wild-type; n = 6 per strain; P = 0.041) and tended to increase in the BMT mice (n = 5, P = 0.058). I, Total CD31+ structure density was decreased in mutants (n = 6) compared with wild-type (n = 6; P = 0.47) and BMT mice (n = 5, P = 0.034). J, BMT reduced scar length in BMT mice (n = 5, P = 0.034) compared with mutant mice (n = 6).

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**Disclosures**

None.

**References**


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