Postinfarct Cytokine Therapy Regenerates Cardiac Tissue and Improves Left Ventricular Function

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Abstract—We systematically investigated the comparative efficacy of three different cytokine regimens, administered after a reperfused myocardial infarction, in regenerating cardiac tissue and improving left ventricular (LV) function. Wild-type (WT) mice underwent a 30-minute coronary occlusion followed by reperfusion and received vehicle, granulocyte colony-stimulating factor (G-CSF)+Flt-3 ligand (FL), G-CSF+stem cell factor (SCF), or G-CSF alone starting 4 hours after reperfusion. In separate experiments, chimeric mice generated by reconstitution of radioablated WT mice with bone marrow from enhanced green fluorescent protein (EGFP) transgenic mice underwent identical protocols. Mice were euthanized 5 weeks later. Echocardiographically, LV function was improved in G-CSF+FL- and G-CSF+SCF-treated but not in G-CSF-treated mice, whereas LV end-diastolic dimensions were smaller in all three groups. Morphometrically, cytokine-treated hearts had smaller LV diameter and volume. Numerous EGFP-positive cardiomyocytes, capillaries, and arterioles were noted in the infarcted region in cytokine-treated chimeric mice treated with G-CSF+FL or G-CSF+SCF, but the numbers were much smaller in G-CSF-treated mice. G-CSF+FL therapy mobilized bone marrow–derived cells exhibiting increased expression of surface antigens (CD62L and CD11a) that facilitate homing. We conclude that postinfarct cytokine therapy with G-CSF+FL or G-CSF+SCF limits adverse LV remodeling and improves LV performance by promoting cardiac regeneration and probably also by exerting other beneficial actions unrelated to regeneration, and that G-CSF alone is less effective. (Circ Res. 2006;98:1098-1105.)

Key Words: myocardial regeneration ■ cytokine ■ stem cell ■ ischemia/reperfusion ■ bone marrow ■ left ventricular function

Despite significant advances in the treatment of acute myocardial infarction (MI), no therapies are currently available to restore dead myocardium. Although mobilization of bone marrow cells (BMCs) by cytokines has been suggested to regenerate cardiac tissue after MI and to produce functional improvement, the reports on the effects of cytokine treatment are conflicting.1–3 Furthermore, previous studies of cytokines have used models of permanent coronary ligation1–3,5 that do not reflect the fact that most patients with acute MI undergo coronary reperfusion. In addition, these studies have administered cytokines as a pretreatment1,2 or performed splenectomy1,2,5 or both,1,2 neither of which would be clinically feasible. Although various cytokines have been tested, the relative efficacy of different treatments or combinations of treatments has not been elucidated. Finally, the mechanism whereby cytokines improve left ventricular (LV) function and structure after MI remains obscure. It has been proposed that cytokines mobilize BMCs with subsequent homing to the infarcted tissue and transdifferentiation into cardiac lineage,1–3 but this hypothesis remains unproven. Thus, numerous unresolved issues persist regarding the use of cytokines as a strategy to achieve cardiac repair after MI.

Accordingly, in the present study, we examined the effect of three cytokines (granulocyte colony-stimulating factor [G-CSF], stem cell factor [SCF], and Flt-3 ligand [FL]) on cardiac repair. The specific goals were to determine: (1) whether cytokine therapy is efficacious in the setting of a reperfused MI; (2) whether it is efficacious when started after the MI and in the presence of an intact spleen; (3) which cytokine or cytokine combination, among G-CSF, FL, and SCF, is more effective in improving LV remodeling and function; (4) whether cytokine-mobilized BMCs home to the heart and transdifferentiate into the cardiac lineage; and (5) whether cytokines facilitate BMC homing to the injured myocardium by altering the expression profile of adhesion molecules in mobilized BMCs. A well-characterized murine model of temporary coronary occlusion followed by reperfusion (a setting designed to mimic the most common clinical
setting of acute MI in humans) was used. A thorough assessment of LV function and anatomy was performed in conjunction with careful measurements of cardiac regeneration by confocal microscopy.

Materials and Methods
A detailed description of all materials and methods is provided in the online supplement, available at http://circres.ahajournals.org.

Generation and Characterization of EGFP Chimeric Mice
EGFP chimeric mice were generated by reconstituting radioablated adult male mice (B6,129 strain) with BMCs from syngeneic enhanced green fluorescent protein (EGFP) transgenic mice. On average, 85% of the peripheral blood nucleated cells were positive for EGFP in the recipients.

Experimental Protocol
Adult male wild-type (WT; B6,129 strain, 10 to 18 weeks of age) and EGFP chimeric mice were used. The overall experimental design is summarized in supplemental Figure I.

Myocardial Infarction
Anesthetized mice underwent a 30-minute occlusion of the left anterior descending coronary artery followed by reperfusion. Starting 4 hours after reperfusion, mice received daily subcutaneous injections of vehicle or cytokine as groups I through IV, respectively, was also assessed by flow cytometry.

Statistical Analysis
Data are reported as mean±SEM. Morphometric and histologic data were analyzed with one-way ANOVA. Serial echocardiographic parameters were measured with a two-way (time and group) ANOVA followed by Student t tests with the Bonferroni correction.

Results
Exclusions
A total of 188 mice (162 WT and 26 EGFP chimeric) were used. One hundred fifty-six mice were assigned to the MI studies (groups I through XII) and 32 mice to the cellular mobilization (groups XIII through XX) studies. Twenty-nine mice died in the perioperative period. Nine mice were excluded from the study because of failure of the coronary occluder, leaving a total of 14, 19, 20, and 18 WT mice in groups I through IV, respectively, and 3, 7, 6, and 5 EGFP chimeric mice in groups V through VIII, respectively. No mouse was excluded from the cellular mobilization studies (groups XIII through XX, n=4 per group).

Myocardial Infarct Size
The impact of cytokine therapy on LV structural and functional parameters was assessed in groups I through IV. The infarct volume fraction was similar in the four groups (supplemental Figure III), indicating that the extent of myocardial cell death produced by the coronary occlusion was comparable. The infarct volume fraction measures the volume of scarred tissue expressed as a fraction of the entire left ventricle.10–12 Because the small islands of regenerated cardiomyocytes scattered throughout the scar area were included in the “infarct” region, in cytokine-treated hearts, the calculation of the infarct volume fraction was not affected by myocardial regeneration.

Assessment of Cardiomyocyte Proliferation
The percentage of cardiomyocyte nuclei positive for 5-bromodeoxyuridine (BrDU) and Ki67 was determined at 14 days after reperfusion in 4 to 6 mice in each of groups IX through XII. In addition, the percentage of cardiomyocyte nuclei positive for Ki67 at 35 days after MI was determined in 6 mice in each of groups I through IV.

Flow Cytometry
After vehicle or cytokine treatment, peripheral blood was harvested from mice in groups XIII through XVI at serial time points, stained with antibodies, and lin- Sca-1/c-kit cells were counted from the lymphoid gate with a multiparameter, live sterile cell sorter.14,15 The flow cytometric procedure is summarized in supplemental Figure II. The expression of adhesion molecules on lin-/Sca-1/c-kit cells from untreated murine bone marrow and peripheral blood of mice (groups XVII through XX) treated with identical dosages of vehicle or cytokines as groups I through IV, respectively, was also assessed by flow cytometry.

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G-CSF+FL and G-CSF+SCF Therapy Improves LV Systolic Function
Before coronary occlusion (baseline), all parameters of LV function, measured by echocardiography, were similar in groups I through IV (Figure 1). At 48 hours after reperfusion, the degree of LV systolic functional impairment did not differ among groups (Figure 1), indicating that the injury sustained during ischemia/reperfusion was comparable. In vehicle-treated mice, there was further functional deterioration between 48 hours and 35 days after reperfusion (Figure 1). In contrast, in G-CSF+FL-treated (group II) and G-CSF+SCF-treated (group III) mice, LV systolic function remained unchanged or improved at 35 days compared with 48 hours (Figure 1). Mice in groups II and III exhibited significantly higher LV ejection fraction, fractional area change, and fractional shortening and lower LV end-systolic diameter compared with vehicle-treated mice (group I) (Figure 1). Compared with group I, myocardial function in the infarct region was greater in groups II and III (systolic thickening
fraction in the infarct wall, 18.6±5.9%, 26.5±2.8%, 24.8±5.4%, and 20.9±6.6% in groups I, II, III, and IV, respectively, but the differences were not statistically significant. Compared with group I, myocardial function was also enhanced in the nonischemic LV region in groups II and III (systolic thickening fraction, 33.2±4.3%, 43.9±5.3%, and 54.4±3.2% [P<0.05 versus group I] and 35.1±4.1% in groups I, II, III, and IV, respectively). In mice treated with G-CSF alone (group IV), there was only a marginal improvement in parameters of LV systolic function (Figure 1E through 1G).

Cytokine Therapy Halts LV Remodeling
Both morphometric and echocardiographic analyses demonstrated that administration of cytokines attenuated adverse LV remodeling 5 weeks after infarction. Morphometrically, LV chamber diameter and LV chamber volume were lower in groups II, III, and IV compared with group I (Figure 2A and 2B; supplemental Figure IV). Echocardiographically, LV anatomical parameters were similar in groups I through IV, both before coronary occlusion (baseline) and 48 hours after reperfusion (Figure 2C and 2D). In vehicle-treated mice (group I), there was significant LV dilatation between 48 hours and 35 days after reperfusion (Figure 2), indicative of adverse LV remodeling. Compared with group I, both echocardiographic (Figure 2C and 2D) and morphometric (supplemental Figure IV) analyses showed smaller LV diameter and LV end-diastolic volume in cytokine-treated mice (groups II through IV). However, the reduction in LV dilatation in group IV was not statistically significant by echocardiography (Figure 2C and 2D), and the reduction in LV volume in group II was not statistically significant by morphometry (supplemental Figure IV). By morphometry, the infarct wall thickness improved in cytokine-treated (groups III and IV) mice (Figure 2E). Interestingly, mice that received G-CSF alone (group IV) also demonstrated a comparable improvement in morphometric parameters of LV remodeling (supplemental Figure IV) despite borderline functional improvement (Figure 1).

Cytokine Therapy Promotes Regeneration of Cardiomyocytes
In EGFP chimeric mice, the effects of cytokines on LV function and anatomy after infarction were directionally concordant with those in WT mice (supplemental Figures V and VI, respectively), despite the fact that LV size was smaller in the chimeric mice (supplemental Figure VI) compared with WT mice (Figure 2). Histologic quantitation of myocardial regeneration was performed in hearts (n=3 to 7 per group) from EGFP chimeric mice (groups V through VIII). Cardiomyocytes derived from BMCs were identified by concomitant positivity for α-sarcomeric actin and EGFP (Figure 3). The extent of regeneration was assessed by measuring the area occupied by EGFP+ myocytes rather than

Figure 1. A through D, Representative 2D (A and C) and M-mode (B and D) images from a vehicle-treated (A and B) and a G-CSF+FL–treated (C and D) mouse 35 days after coronary occlusion/reperfusion. Compared with the vehicle-treated heart, the G-CSF+FL–treated heart exhibits a smaller LV cavity and thicker infarct wall. Contractile activity in the infarct area is present in the G-CSF+FL–treated mouse (arrowheads) and absent in the vehicle-treated mouse. E through G, Echocardiographic assessment of LV systolic function. Echocardiographic measurements in control and cytokine-treated mice (groups I through IV) are illustrated. The administration of G-CSF+FL and G-CSF+SCF improved LV systolic function 35 days after MI. Data are mean±SEM. n=14 to 19 mice per group. *P<0.05 vs group I.
their number; the former parameter more accurately reflects the functional impact of regeneration. In the infarcted region, EGFP\textsuperscript{+} regenerated cardiomyocytes occupied only 0.9±0.1% of the myocardial area in group I; in contrast, this fraction of reconstituted myocardium was much higher in groups II, III, and IV (14.9±1.5%, 10.8±1.9%, and 4.7±1.0% of the myocardial area, respectively). The area occupied by regenerated cardiomyocytes was similar in the infarct and in the border zone. In contrast, in the noninfarcted region, the area occupied by EGFP\textsuperscript{+} myocytes was uniformly low in all four groups (0.2±0.1%, 0.6±0.3%, 0.4±0.1%, and 0.4±0.1% of myocardial area in groups I, II, III, and IV, respectively; Figure 4). Newly formed myocytes expressed connexin 43 (Figure 5), indicating functional integration with adjacent myocytes. EGFP\textsuperscript{−} cells that were negative for cardiomyocyte markers constituted 1.2±0.6%, 3.9±0.6%, 3.5±0.6%, and

Figure 2. Cytokine therapy and postinfarct LV remodeling. Representative Masson’s trichrome-stained myocardial sections from a control (A) and a G-CSF+FL–treated (B) WT mouse. Scar tissue and viable myocardium are identified in blue and purple, respectively. The LV cavity is smaller, and the infarcted as well as the noninfarcted walls are thicker in the G-CSF+FL–treated mouse compared with the control mouse. Panels C through E illustrate measurements of LV structural parameters in diastole by echocardiography (C and D) and morphometry (E) in groups I through IV. Administration of G-CSF+FL and G-CSF+SCF attenuated adverse LV remodeling and improved cardiac anatomy, whereas G-CSF alone had marginal effects. Data are mean±SEM. n=14 to 19 mice per group. *P<0.05 vs group I.

Figure 3. Postinfarct cytokine therapy induces cardiomyocyte regeneration in EGFP chimeric mice. BMCs and myocytes are identified by EGFP (green) and α-sarcomeric actin (red), respectively. A through C, Low-magnification images of the infarct area in a vehicle-treated mouse show a small number of EGFP\textsuperscript{+} BMCs and rare transdifferentiation into myocytes (C, yellow, arrowhead). D through F, In contrast, images of the infarct area in a G-CSF+FL–treated mouse show numerous small myocytes (F, yellow) that are positive for both EGFP and α-sarcomeric actin. Arrowheads indicate small myocytes that are EGFP\textsuperscript{−}, possibly derived from resident stem cells. G through I, Higher magnification images of the infarct area in a G-CSF+FL–treated mouse show several newly formed myocytes (I, yellow, arrowheads) positive for EGFP and α-sarcomeric actin. Nuclei are stained with 4',6-diamidino-2-phenylindole (blue). Bar=20 μm.
Cardiomyocyte Proliferation

The effect of cytokine administration on cardiomyocyte proliferation at 14 days after MI was assessed in groups IX through XII by examining BrdU incorporation (Figure 6A and 6C). In addition, 6 hearts from each of groups I through IV were examined for Ki67 positivity at 35 days after MI (Figure 6D). The increase in Ki67+ cardiomyocytes in the infarcted region, indicating increased numbers of cycling myocytes (Figure 6). The increase observed with G-CSF alone was significantly less than that seen with G-CSF + FL and G-CSF + SCF. In contrast, the number of cycling myocytes positive for Ki67 in the nonischemic zone at 35 days after MI was comparable in all groups (data not shown).

Regeneration of the Vasculature

Colocalization of EGFP positivity with α-smooth muscle actin positivity in arteriolar smooth muscle cells (Figure 7A) and PECAM-1 positivity in endothelial cells (Figure 7B) were noted predominantly in the infarct area and, to a lesser extent, in the viable myocardium. Thus, EGFP+ BMCs differentiated not only into cardiomyocytes but also into vascular structures necessary to supply blood to the newly formed myocardium in the infarcted region.

Mobilization of Lin/Sca-1+/c-kit+ BMCs

The efficacy of different cytokine regimens in mobilizing Lin-/Sca-1-/c-kit+ BMCs was examined by flow cytometry in groups XIII through XVI. Compared with groups XIII, XV, and XVI, G-CSF+FL (group XIV) therapy resulted in mobilization of a significantly higher number of Lin-/Sca-1-/c-kit+ BMCs (0.1±0.1, 28.8±3.9, 5.8±1.3, and 4.0±0.7 cells/µL at 5 days in groups XIII, XIV, XV, and XVI, respectively). The increase in Lin-/Sca-1-/c-kit+ BMC levels in group XIV was apparent at 3 days of therapy and became progressively more pronounced at 5 and 10 days.
G-CSF+SCF (group XV) and G-CSF alone (group XVI) resulted in minimal changes in peripheral blood levels of lin/Sca-1+/c-kit+ cells.

### Cytokine Therapy Enhances Expression of Surface Antigens That Favor Homing

In groups XVII through XX, peripheral blood cells were analyzed by flow cytometry for expression of adhesion molecules on mobilized lin/Sca-1+/c-kit+ BMCs. As shown in supplemental Figure 7, all three treatments (G-CSF+FL, G-CSF+SCF, and G-CSF alone) resulted in increased expression of CD62P (P-selectin), an adhesion molecule that favors attachment and extravasation of BMCs,16 and decreased expression of CD49d, an adhesion molecule that plays a critical role in retaining cells within the bone marrow.17 Both of these changes would be expected to promote release of BMCs from bone marrow and their migration into the cardiac interstitium. However, there were significant differences in adhesion molecule profile between G-CSF+FL-treated mice on the one hand and G-CSF+SCF- and G-CSF-treated mice on the other hand. Specifically, G-CSF+SCF and G-CSF treatment reduced whereas G-CSF+FL treatment increased the expression of CD62L (L-selectin) and CD11a (Figure 8), two adhesion molecules that favor extravasation and homing of BMCs.16–18 Furthermore, the reduction in expression of CD49d, an adhesion molecule that promotes BMC attachment,17 was comparatively less with G-CSF+FL and G-CSF+SCF treatment vis-à-vis G-CSF alone (supplemental Figure VII). In conjunction with the quantitative analysis of the number of circulating lin/Sca-1+/c-kit+ cells reported above, these results demonstrate significant differences among different cytokine regimens with respect to the modulation of the adhesion molecules responsible for homing.

### Discussion

Most basic and clinical studies of cardiac regeneration have focused on cell-based therapies,4 and relatively less is known regarding the role of cytokines in this process. The present investigation provides a number of important new findings. Our results demonstrate that: (1) cytokine therapy with a combination of G-CSF+FL or G-CSF+SCF started after acute MI, halts or attenuates adverse LV remodeling, and improves LV systolic function; (2) cytokine therapy results in mobilization of bone marrow stem cells (lin/Sca-1+/c-kit+), which home to the injured myocardium and transdifferentiate into the cardiac lineage (cardiomyocytes, vascular smooth muscle cells, endothelial cells); (3) the combination of G-CSF and FL is superior to G-CSF+SCF or G-CSF alone with respect to its effects on LV remodeling, LV function, and mobilization of lin/Sca-1+/c-kit+ BMCs into the peripheral blood; (4) all three cytokine regimens upregulated the expression of CD62P and downregulated the expression of CD49d on BMCs (CD62P [P-selectin] promotes the extravasation of BMCs, whereas CD49d is important for the retention of these cells in the marrow16,17); and (5) in contrast to G-CSF+SCF and G-CSF alone, G-CSF+FL treatment increases the expression of adhesion molecules (CD62L and CD11a) that favor homing of BMCs,16–18 CD62L (L-selectin) and the β2 integrin leukocyte function antigen-1 (CD11a) initiate and facilitate the attachment of peripheral blood cells to the endothelium, thereby enhancing the extravasation of these cells,17,18. Thus, the present investigation not only establishes the usefulness of cytokine therapy for therapeutic cardiac regeneration but also provides mechanistic insights into this phenomenon and identifies the superiority of a specific cytokine combination (G-CSF+FL). These results have important implications for the role of cytokine-based strategies in effecting cardiac repair after acute MI.

Previous studies have shown that cytokine therapies initiated before1,2 or after3 MI result in improved LV remodeling and function. However, to our knowledge, this is the first study to demonstrate that cytokine administration results in transdifferentiation of mobilized BMCs into the cardiac lineage and in expression of adhesion molecules that favor homing of BMCs to the injured myocardium. Furthermore, this is the first investigation to systematically compare the efficacy of G-CSF, FL, and SCF and their combination on postinfarct LV remodeling and function, and to identify the combination of G-CSF and FL as the most effective treatment.

A number of methodological considerations are in order. In contrast to previous studies, which examined models of permanent coronary occlusion,1 we elected to use a transient...
(30-minute) coronary occlusion followed by reperfusion. This model has greater clinical relevance because most patients with acute MI undergo either spontaneous or therapeutic coronary recanalization. We chose to initiate cytokine treatment after the MI because pretreatment (as used in previous studies) would not be clinically feasible. For similar reasons, we elected not to perform a splenectomy (which was performed in previous investigations). The doses of cytokines used in this study were selected because they are clinically relevant. The large group sizes (14 to 20 mice per group in groups I through IV) were deemed to be important to achieve robust conclusions. In an effort to perform a rigorous assessment of global as well as regional LV function, we used multiple echocardiographic parameters, including fractional shortening (parasternal short-axis M-mode images), fractional area change (parasternal short-axis 2-D images), ejection fraction, and systolic wall thickening. Treatment with G-CSF + FL or G-CSF + SCF consistently improved all of these parameters, providing solid evidence for the functional benefit of these therapies. This concept is further corroborated by the fact that two independent techniques (echocardiography and morphometry, performed by blinded investigators who were unaware of the data obtained with the other technique) demonstrated that G-CSF + FL and G-CSF + SCF were effective in mitigating the progressive LV chamber dilatation that is characteristic of adverse LV remodeling and ischemic cardiomyopathy. The data obtained by these two methods were generally concordant. However, in Figure 2E, we present the morphometric infarct wall thickness data because the measurement of the average integrated wall thickness by morphometry may be inherently more accurate.

We chose to examine G-CSF, SCF, and FL because these cytokines are used clinically (G-CSF) or have been well characterized with respect to BMC mobilization. Comparison of different cytokines or combinations thereof is important because these proteins are known to preferentially mobilize different subsets of BMCs. FL was not studied alone because, unlike G-CSF, it does not expand lin-/Sca-1+ c-kit+ cells. SCF was not studied as single therapy because it has low efficacy in mobilizing BMCs when used alone. FL and G-CSF together resulted in mobilization of much higher numbers of lin-/Sca-1+/c-kit+ cells than the lin-/Sca-1+/c-kit+ cells mobilized by G-CSF alone. SCF was not studied as single therapy because it has low efficacy in mobilizing BMCs when used alone.

Our results indicate that the combination of G-CSF and FL produced the most robust results with respect to myocyte regeneration, LV remodeling, LV function, and mobilization of lin-/Sca-1+/c-kit+ cells. G-CSF + SCF also resulted in significant beneficial effects, albeit to a lesser extent. The most plausible explanation for the superiority of G-CSF + FL is that this combination resulted in mobilization of much higher numbers of lin-/Sca-1+/c-kit+ cells into the peripheral blood, which would be expected to enhance the homing and engraftment of BMCs in the damaged myocardium. In addition to these quantitative differences, our flow cytometric analysis reveals qualitative differences; that is, the lin-/Sca-1+/c-kit+ cells mobilized by G-CSF + FL expressed higher levels of CD62L and CD11a vis-à-vis the cells mobilized by G-CSF + SCF (Figure 8). The expression of these adhesion molecules may facilitate the egress of primitive BMCs from the circulation into injured tissues that express chemoattractants.

Having established the functional and structural benefits of cytokine therapy, we used EGFP chimeric mice to investigate the mechanism for these salutary actions. Our results demonstrate that after administration of G-CSF + FL or G-CSF + SCF, mobilized BMCs home to the infarcted myocardium and differentiate into cardiomyocytes, smooth muscle cells, and endothelial cells. We found that cardiomyocyte regeneration was considerably greater with G-CSF + FL or G-CSF + SCF versus G-CSF alone (Figure 4). These findings were independently validated by the measurement of cycling (i.e., BrdU+ and Ki67+) cardiomyocytes in non-EGFP chimeric mice (Figure 6).

The mechanism(s) whereby cytokines produce beneficial effects on LV dimensions and function after MI is likely complex. Although the association of greater mobilization of lin-/Sca-1+/c-kit+ cells with greater improvement in LV remodeling and function after G-CSF + FL treatment suggests that these cells may play an important role in cardiac regeneration, our results do not prove that the beneficial effects of cytokines were attributable to this subset of BMCs. The BMCs responsible for cardiac regeneration may be cell types other than the lin-/Sca-1+/c-kit+ cells (eg, multipotent progenitors, endothelial progenitors, etc.). It is also unknown whether, within the lin-/Sca-1+/c-kit+ subset of BMCs, the transdifferentiation was attributable to hematopoietic or nonhematopoietic cells. We recently identified a CXCR4+/Sca-1+/lin-CD45+ nonhematopoietic subfraction of BMCs that appear to be committed to differentiation into cardiac lineages and are mobilized after MI in mice as well as in humans. It is possible that these cells may account for the salutary actions of G-CSF + FL and G-CSF + SCF noted in this study. Finally, mobilization of resident cardiac stem cells by cytokines could be an additional or alternative mechanism. In support of this hypothesis, we noted small cardiomyocytes in the infarct area of cytokine-treated hearts that were not positive for EGFP (Figure 3F, arrowheads), suggesting that cytokine treatment may have induced migration, differentiation, and possibly proliferation of resident cardiac progenitor cells, which may have contributed to cardiac repair.

Harada et al recently concluded that administration of G-CSF ameliorates LV remodeling and dysfunction by inhibiting apoptosis, not by promoting cardiac regeneration. Our results differ because they document transdifferentiation of BMCs into cardiac lineage (Figures 3, 4, and 7; predominantly in the infarct area). Nevertheless, we also noted functional improvement in the noninfarcted myocardium, where the extent of myocyte regeneration was minimal (Figure 4). This enhancement in contractility in the noninfarcted region is likely attributable to nonregenerative actions of cytokines (eg, inhibition of apoptosis, favorable changes in the interstitium, etc.), suggesting that multiple mechanisms contributed to the overall improvement in LV function after G-CSF + FL and G-SCF + SCF therapy. Although G-CSF alone induced little cardiomyocyte regeneration, it had favorable effects on LV remodeling (Figure 2C and 2D; supplemental Figure IV); however, G-CSF alone did not improve LV function significantly (Figure 1). As shown in Figure 1, measurements of LV systolic function were greater in group...
IV than in vehicle-treated mice, but the differences were not statistically significant. Despite the large sample sizes (n=15 to 19 per group), this could be attributable to a type II error. Alternatively, it is possible that other nonregenerative beneficial effects of G-CSF on the myocardium resulted in an improvement in LV anatomy which, however, did not result in improved function because of the less efficient regeneration of cardiomyocytes with G-CSF therapy.

In conclusion, we demonstrated that a clinically relevant protocol for cytokine therapy exerts profound beneficial effects on postinfarction cardiomyopathy. Specifically, administration of G-CSF + FL or G-CSF + SCF, started after a reperfused acute MI, results in regeneration of cardiomyocytes and coronary vessels by mobilized BMCs and in improvement in LV remodeling and function via myocyte regeneration and probably via other beneficial actions of cytokines. We also demonstrated that the combination of G-CSF + FL is superior to G-CSF + SCF or G-CSF alone. These results have significant therapeutic implications. Recent studies with G-CSF have concluded that this cytokine improves LV function and structure in patients with acute MI. Our findings suggest that the combination of G-CSF and FL should have even greater efficacy in the clinical arena, providing a rationale for testing G-CSF + FL in larger species and in patients.

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References

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MATERIALS AND METHODS (supplemental)

Generation and characterization of EGFP chimeric mice. Recipient adult male mice (B6,129 strain, age 8-9 wk, body wt. 20-30 g) were conditioned with 950 cGy total body irradiation from a cesium source (Gamma-cell 40; Nordion, Ontario, Canada). Donor BM was prepared with a modification of a previously described method [1]. Briefly, syngeneic EGFP transgenic donor mice (B6 strain) in which EGFP was driven by the β-actin promoter in all tissues were euthanized, and tibias and femurs were harvested. The BM was expelled from the bones with Medium 199 (GIBCO-BRL, Grand Island, NY) containing 10 µg/ml gentamicin (GIBCO-BRL), referred to hereafter as chimeric medium (CM). The BM was resuspended in CM by gentle aspiration through an 18-gauge needle. The cells were filtered through sterile nylon mesh with 100-µ pores, centrifuged at 1,000 rpm for 10 min at 4°C, and resuspended in CM. A cell count was performed, and the cells were diluted to the appropriate concentration to allow injection of 1 ml per animal. At least 4 h after irradiation, animals underwent transplantation with 1 ml CM containing 1×10^6 BMCs through a lateral tail vein injection using a 27-gauge needle. After BM transplant, recipients were characterized for chimerism by determining the relative percentages of hematopoietic stem cell-derived lineages in the peripheral blood. Peripheral blood was obtained through tail vein bleeding and nucleated cells were counted under a fluorescent microscope to determine the percentage of EGFP-positive cells. On average, 85% of the peripheral blood nucleated cells were positive for EGFP.

Experimental protocol. Adult male wild-type (WT, B6,129 strain, age 10-18 wk, body wt. 20-35 g) and EGFP chimeric mice were used. The overall experimental design is summarized in supplemental Fig. 1. WT (groups I-IV, IX-XII) and chimeric (groups V-VIII) mice underwent a 30-min coronary occlusion followed by reperfusion and received
daily subcutaneous injections of cytokines or vehicle starting 4 h after the onset of reperfusion as follows: groups I, V, and IX, vehicle (days 1-10); groups II, VI, and X, G-CSF (250 µg/kg/d, days 1-5) + FL (333 µg/kg/d, days 1-10); groups III, VII, and XI, G-CSF (250 µg/kg, days 1-5) + SCF (200 µg/kg, days 1-5); and groups IV, VIII, and XII, G-CSF (250 µg/kg, days 1-5). All mice in groups I-IV underwent echocardiographic studies two days prior to coronary occlusion/reperfusion and 48 h and 35 d after reperfusion. Mice in groups I-VIII were sacrificed 35 days after coronary reperfusion. Mice in groups IX-XII received BrdU in drinking water starting 4 h after reperfusion and were sacrificed after 14 days for assessment of cardiomyocyte proliferation. Mice in groups XIII-XX received subcutaneous injections of vehicle (groups XIII and XVII) or cytokines (G-CSF+FL, groups XIV and XVIII; G-CSF+SCF, groups XV and XIX; and G-CSF, groups XVI and XX) and were sacrificed after 10 days for the assessment of BMC mobilization (groups XIII-XVI) and for the analysis of surface antigen expression (groups XVII-XX) by flow cytometry.

**Myocardial infarction.** Male mice (B6,129 strain, age 10-18 wk, body wt. 20-35 g) were obtained from the Jackson Laboratories (Bar Harbor, Maine) and housed under specific pathogen-free conditions. Mice were premedicated with atropine sulfate (0.04 mg/kg im), anesthetized 5 min later with pentobarbital sodium (60 mg/kg iv), intubated with a PE-60 tubing, and ventilated. Additional doses of pentobarbital were given during the protocol as needed to maintain anesthesia. Before surgery started, mice were given gentamicin (0.7 mg/kg im). Body temperature was carefully monitored with a rectal probe and was maintained as close as possible to 37.0°C throughout the experiment by using a heating pad and heat lamps [2]. To replace blood losses, blood from a donor mouse was given intravenously [2].
With the aid of a dissecting microscope and a microcoagulator, the chest was opened through a midline sternotomy. An 8-0 nylon suture was passed with a tapered needle under the left anterior descending coronary artery 2-3 mm from the tip of the left auricle, and a nontraumatic balloon occluder was applied on the artery. Coronary occlusion was induced by inflating the balloon occluder. Successful performance of coronary occlusion and reperfusion was verified by visual inspection (i.e., by noting the development of a pale color in the distal myocardium on inflation of the balloon and the return of a bright red color due to hyperemia after deflation) and by observing S-T segment elevation and widening of the QRS on the ECG during ischemia and their resolution after reperfusion. After the coronary occlusion-reperfusion protocol was completed, the chest was closed in layers, and a small catheter was left in the thorax for 10-20 min to evacuate air and fluids. The mice were removed from the ventilator, kept warm with heat lamps, given fluids (1.0-1.5 ml of 5% dextrose in water intraperitoneally), and allowed 100% oxygen via nasal cone.

**Echocardiographic studies.** Serial echocardiograms were obtained at baseline (2 days prior to coronary occlusion) and 48 h and 35 days after reperfusion using an HDI 5000 SonoCT echocardiography machine (Philips Medical Systems) equipped with 15-7 MHz linear broadband and 12-5 MHz phased array transducers. Prior to echocardiography, mice were weighed and anesthetized with i.p. injection of pentobarbital (25 mg/kg). The anterior chest was shaved and mice were placed in the left lateral decubitus position. A rectal temperature probe was placed and the body temperature was carefully maintained close to 37.0°C with a heating pad throughout the study. The modified parasternal long-axis and parasternal short-axis views were used to obtain two-dimensional (2-D), M-mode, and spectral Doppler images [3]. Systolic and diastolic anatomic parameters were
obtained from M-mode tracings at the mid-papillary level. Systolic thickening fraction was calculated as the ratio of systolic thickening to end-diastolic thickness x 100. LV cross-sectional area was determined from the parasternal short-axis 2-D image. LV volume was estimated by the Teichholz formula. Analysis of data was performed by an investigator who was blind to the treatment assigned and was unaware of data from other modalities.

**Morphometric and histologic analyses.** At the end of the follow-up period, the thorax was opened, the abdominal aorta was cannulated, and the heart was arrested in diastole with KCl and CdCl₂, excised, and perfused retrogradely through the aorta with 10% neutral-buffered formalin. The right atrium was cut to allow drainage. The perfusion pressure was adjusted to match the mean arterial pressure. The LV chamber was filled with fixative from a pressure reservoir set at a height equivalent to the in vivo measured LVEDP [3,4]. After measuring the major longitudinal intracavitary axis, the LV was sectioned serially into four rings perpendicular to its longitudinal axis, processed, and embedded in paraffin. The infarct volume fraction was calculated by computerized planimetry (Image-Pro Plus, Media-Cybernetics, Carlsbad, CA) of digital images of three Masson’s trichrome-stained serial LV sections taken at 0.5-1.0 mm intervals along the longitudinal axis [5]. The mid-section was used to measure LV diameter and volume [6,7]. The thickness of the infarct wall, septal wall, and posterior wall was calculated in serial sections and averaged [6,7]. An average sarcomere length of 2.1 µm was utilized in all cases to correct the raw measurements of LV anatomical parameters [8]. Analysis of data was performed by an investigator who was blind to the treatment assigned and was unaware of data from other modalities.
Immunohistochemistry was performed in formalin-fixed 4-µm-thick histological sections. Cardiomyocytes were recognized by the presence of α-sarcomeric actin and troponin T; endothelial cells by PECAM-1 and von Willebrand factor; and smooth muscle cells by α-smooth muscle actin. Cycling cells were detected with BrdU and Ki67 antibodies. Colocalization of cell-specific markers with EGFP was used to identify cells that originated from BMCs. Nuclei were identified with DAPI or propidium iodide (PI) [3,4]. All of the primary and secondary antibodies utilized in these studies are specified in supplemental Table 1. A quantitative analysis of cardiomyocyte regeneration was performed in 3-7 EGFP chimeric mouse hearts in each of groups V-VIII. Serial LV sections through the infarct region were used and an average of 100 fields (196x196 µ) were examined in each section. For each parameter examined, the sampling area ranged between 3 and 5 mm²/section.

**Assessment of cardiomyocyte proliferation.** Myocardial sections were stained for BrdU and Ki67 [3] with the antibodies specified in supplemental Table 1. Cardiomyocytes were recognized by positivity for α-sarcomeric actin. The percentage of cardiomyocyte nuclei positive for BrdU and Ki67 at 14 days after reperfusion was determined in 4-6 mice in each of groups IX-XII. In addition, the percentage of cardiomyocyte nuclei positive for Ki67 at 35 d after MI was determined in 6 mice in each of groups I-IV.

**Flow cytometric analysis.** WT mice in groups XIII-XVI received the same dose of vehicle or cytokines as in groups I-IV, respectively. Peripheral blood was harvested at serial time points and lin⁻/Sca-1⁺/c-kit⁺ cells were positively selected with a multiparameter, live sterile cell sorter (FACSVantage SE; Becton Dickinson,
Mountainview, CA) [9]. Briefly, peripheral blood was collected from donor mice and filtered, centrifuged, washed, and resuspended in sterile cell sort medium (CSM). Directly labeled monoclonal antibodies (mAbs, Pharmingen) were added at saturating concentrations: Sca-1 PE, c-kit PE-Cy5, CD8α APC, Mac-1 APC, B220 APC, Gr-1 APC, and β-TCR APC. The cells were incubated at 4°C for 30 min, washed twice in CSM, and resuspended. Lin−/Sca-1+/c-kit+ cells were counted from the lymphoid gate [10]. The flow cytometric procedure is summarized in supplemental Fig. 2.

The expression of adhesion molecules on lin−/Sca-1+/c-kit+ cells from untreated murine BM and peripheral blood of mice (groups XVII-XX) treated with identical dosages of vehicle or cytokines as groups I-IV, respectively, was assessed by flow cytometry. BMCs or peripheral blood cells were stained for lineage markers, Sca-1, c-kit, and adhesion molecules using monoclonal antibodies (mAbs) against CD54, CD44, CD62P, CD49D, CD62L, CD11a, CD49e, CD102, or CD106, or the appropriate isotype control mAbs. The experiments were performed in duplicate and a minimum of 100,000 total events was collected from each sample for each experiment.

**Statistical analysis.** Data are reported as mean±SEM. Morphometric and histologic data were analyzed with one-way ANOVA [11]. Serial echocardiographic parameters were measured with a two-way (time and group) ANOVA followed by Student’s t-tests with the Bonferroni correction [11]. All statistical analyses were performed using the SPSS software (version 8, SPSS, Inc., Chicago, IL).
REFERENCES


### Supplemental Table 1. Primary and secondary antibodies used.

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IHC, immunohistochemistry
Supplemental Figure 1. Experimental protocol. Twenty groups of mice were used. Four days after a baseline echocardiogram, WT mice (groups I-IV, n=14-20/group) and EGFP chimeric mice (groups V-VIII, n=3-7/group) underwent a 30-min coronary occlusion followed by reperfusion. Starting 4 h after MI, mice received daily subcutaneous injections of vehicle (groups I and V), G-CSF+FL (groups II and VI), G-CSF+SCF (groups III and VII), or G-CSF alone (groups IV and VIII). Echocardiography was repeated at 48 h and 35 d after MI. At 35 d after MI, mice were sacrificed for morphometric and histologic studies. WT mice in groups IX-XII (n=6/group) underwent a 30-min coronary occlusion followed by reperfusion and received the same therapy as above with vehicle (group IX), G-CSF+FL (group X), G-CSF+SCF (group XI), or G-CSF alone (group XII). In addition, groups IX-XII received BrdU (1 mg/L) in drinking water ad lib and were sacrificed 14 d after MI for the studies of myocyte proliferation. Mice in groups XIII-XVI (n=4/group) received vehicle or cytokine injection as above, and were sacrificed after 10 d. Peripheral blood was collected at serial time-points and mobilization of lin⁻/Sca-1⁻/c-kit⁺ cells was analyzed by flow cytometry. Mice in groups XVII-XX (n=4/group) received vehicle or cytokine treatment as above and were sacrificed after 10 d for the analysis of adhesion molecule expression in lin⁻/Sca-1⁺/c-kit⁺ cells by flow cytometry.

Supplemental Figure 2. Flow cytometric assessment of BMC mobilization. BMCs from B6,129 mice treated with vehicle or cytokine combinations (groups XIII-XVI) were stained with monoclonal antibodies against Sca-1, c-kit, and lineage markers. Lineage negative cells were further gated for Sca-1⁺ and c-kit⁺ cells (R3).
Supplemental Figure 3. Myocardial infarct size in groups I-IV (treated with vehicle, G-CSF+FL, G-CSF+SCF, and G-CSF, respectively). O, Individual mice; ●, mean±SEM; n, number of mice.

Supplemental Figure 4. Morphometric assessment of LV anatomy in wild-type mice (groups I-IV). The two panels illustrate LV chamber diameter and chamber volume measured postmortem in hearts arrested in diastole. Treatment with G-CSF+FL, G-CSF+SCF, or G-CSF alone attenuated adverse LV remodeling. Data are mean±SEM. n=15-19 mice/group. *P < 0.05 vs. group I.

Supplemental Figure 5. Echocardiographic assessment of LV systolic function in EGFP chimeric mice (groups V-VIII). The administration of G-CSF+FL and G-CSF+SCF improved LV systolic function 35 d after MI. Data are mean±SEM. n=3-7 mice/group. *P < 0.05 vs. group V; #P < 0.05 vs. group VIII.

Supplemental Figure 6. Morphometric assessment of LV anatomy in EGFP chimeric mice (groups V-VIII). The three panels illustrate LV chamber diameter, chamber volume, and diastolic infarct wall thickness measured postmortem in hearts arrested in diastole. Treatment with G-CSF+FL, G-CSF+SCF, or G-CSF alone attenuated adverse LV remodeling. Data are mean±SEM. n=3-7 mice/group. *P < 0.05 vs. group V; #P < 0.05 vs. group VII.

Supplemental Figure 7. Flow cytometric assessment of adhesion molecule expression in lin−/Sca-1+/c-kit+ cells from untreated BM and peripheral blood of mice treated with G-CSF+FL, G-CSF+SCF, or G-CSF alone. Shown is the overlay of the adhesion molecules (red) and the isotype control (green). Each experiment was performed in duplicate. A
minimum of 100,000 total events was collected from each sample for each histogram. Treatment with G-CSF+FL and G-CSF+SCF induced the expression of an adhesion molecule profile conducive to homing. BM, bone marrow; PB, peripheral blood.
**GROUPS I-IV**

- Daily s.c. vehicle or cytokine injection
- Days 1-10
- Assessment of myocyte proliferation
- Morphometry and histology

**GROUPS V-VIII**

- Daily s.c. vehicle or cytokine injection
- Days 1-10
- Assessment of cardiac regeneration

**GROUPS IX-XII**

- Daily s.c. vehicle or cytokine injection
- Days 1-10
- Assessment of myocyte proliferation

**GROUPS XIII-XVI**

- Daily s.c. vehicle or cytokine injection
- Days 1-10
- Flow cytometric assessment of BMC mobilization

**GROUPS XVII-XX**

- Daily s.c. vehicle or cytokine injection
- Days 1-10
- Flow cytometric analysis of adhesion molecule expression

**SUPPLEMENTAL FIGURE 1**
SUPPLEMENTAL FIGURE 2
Supplemental Figure 3
SUPPLEMENTAL FIGURE 4

A. LV Chamber Diameter

B. LV Chamber Volume
SUPPLEMENTAL FIGURE 5

Graphs showing data for different groups:

A. Ejection Fraction
B. Fractional Area Change
C. End-Systolic Diameter

Legend:
- Group V (Vehicle)
- Group VI (G-CSF+FL)
- Group VII (G-CSF+SCF)
- Group VIII (G-CSF)

Significance markers:
- * for Group VI
- # for Group VII
- ** for Group VIII

Bar charts indicate changes in Ejection Fraction, Fractional Area Change, and End-Systolic Diameter across different groups.
SUPPLEMENTAL FIGURE 6

A. LV Chamber Diameter

B. LV Chamber Volume

C. Infarct Wall Thickness (diastole)