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The Beneficial Effects of Postinfarct Cytokine Combination Therapy are Sustained During Long-Term Follow-Up

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Abstract

We have previously reported that administration of granulocyte colony-stimulating factor (G-CSF) +Flt-3 ligand (FL) or G-CSF+stem cell factor (SCF) improves left ventricular (LV) function and halts LV remodeling at 35 days after myocardial infarction (MI). In the current study, we investigated whether these beneficial effects are sustained in the long term - an issue of fundamental importance for clinical translation. Mice undergoing a 30-min coronary occlusion followed by reperfusion received vehicle (group I), G-CSF+FL (group II), G-CSF+SCF (group III), or G-CSF alone (group IV) starting 4 h after reperfusion and were euthanized 48 weeks later. LV structure and function were assessed by serial echocardiography before and at 48 h and 4, 8, 16, 32, and 48 wk after MI. During follow-up, mice in group I exhibited worsening of LV function and progressive LV remodeling. Compared with group I, both groups II and III exhibited improved LV EF at 4 wk after MI; however, only in group II was this improvement sustained at 48 wk. Group II was also the only group in which the decrease in infarct wall thickening fraction, the LV dilatation, and the increase in LV mass were attenuated vs. group I. We conclude that the beneficial effect of G-CSF+FL on postinfarction LV dysfunction and remodeling is sustained for at least 11 months, and thus is likely to be permanent. In contrast, the effect of G-CSF+SCF was not sustained beyond the first few weeks, and G-CSF alone is ineffective. To our knowledge, this is the first long-term study of cytokines in postinfarction LV remodeling. The results reveal heretofore unknown differential actions of cytokines and have important translational implications.

Keywords

myocardial repair; cytokine; stem cell; ventricular remodeling; heart failure; bone marrow

Introduction

Although prompt restoration of perfusion with thrombolysis and interventional strategies has significantly decreased the early morbidity and mortality from myocardial infarction (MI), post-infarction heart failure resulting from progressive left ventricular (LV) remodeling is

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reaching epidemic proportions. Recent reports indicate that therapy with hematopoietic cytokines, including granulocyte colony-stimulating factor (G-CSF), stem cell factor (SCF), Flt-3 ligand (FL), erythropoietin, and leukemia inhibitory factor can promote cardiac repair and improve remodeling after MI [1-4]. The mechanisms underlying these observed benefits remain unclear; however, homing of cytokine-mobilized bone marrow cells (BMCs) into the infarcted myocardium with consequent regeneration of myocytes and vasculature is thought to play an important role in this process [1,2,5]. In this regard, although improvement in LV function following BMC therapy has been documented during short follow-up in numerous animal and human studies [6], it has been suggested that the paracrine beneficial effects of BMC therapy on cardiac structure and function may disappear after a few months [7,8]. No previous study has examined the long-term effects of cytokine therapy. From a translational standpoint, it is therefore critically important to examine whether the cardiac reparative benefits of cytokine therapy are sustained during a long follow-up.

Another important unresolved issue pertaining to cytokine-mediated infarct repair is the comparative efficacy of different cytokines. We previously reported that the administration of cytokine combinations (G-CSF+FL and G-CSF+SCF) after acute MI results in significant improvement in LV function and remodeling during a relatively short (35 d) follow-up; however, G-CSF alone is not effective in this setting [2]. Consistent with our observations, therapy with G-CSF alone in patients with acute MI has resulted in variable outcomes [9-14], and two recent meta-analyses of randomized controlled clinical studies of G-CSF therapy in unselected patients with acute MI showed no significant improvement compared with controls [15,16]. However, in our meta-analysis G-CSF therapy was associated with improvement in LV function in patients with impaired LV ejection fraction (EF) at baseline [16]. In view of these disparate results, and given the virtual absence of data on the comparative effects of cytokines, it seems important to perform a careful and direct comparison of various cytokine regimens over long follow-up periods.

Accordingly, the goals of the current study were: i) to determine whether the effects of cytokine combinations (G-CSF+FL and G-CSF+SCF) and G-CSF alone on LV function and remodeling after myocardial ischemia/reperfusion injury in mice are sustained over an 11-month follow-up, a period significantly longer than the 35-day follow-up previously examined; and ii) to examine which cytokine or cytokine combination confers greater benefits during such an extended follow-up. A well established mouse model of ischemia/reperfusion injury was used [2,17,18]. In order to compare our present data with our previous results [2], we utilized the same animal model and the same doses of cytokines as in that study. The rationale for studying G-CSF+FL, G-CSF+SCF, and G-CSF alone has been laid out previously [2]. Given the extensive duration of this study, examining additional cytokine regimens would have been practically infeasible. Our results indicate that the improvement in LV function following administration of G-CSF+FL is sustained for at least 11 months, while therapy with G-CSF+SCF or G-CSF alone is ineffective over this time frame. These results may have important implications for the design of future studies of cytokine therapy for cardiac repair in humans.

Materials and Methods

Experimental protocol

Male ICR mice (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. The overall experimental design is summarized in Fig. 1. 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: group I, vehicle (days 1-10); group II, G-CSF (250 µg/kg/d, days 1-5) + FL (333 µg/kg/d, days 1-10); group III, G-CSF (250 µg/kg, days 1-5) + SCF (200 µg/kg, days 1-5); and group IV, G-CSF (250 µg/kg, days 1-5). In rodents,

the half-lives of G-CSF and SCF have been shown to be 2-4 h and 2 h, respectively [19-21] (authors' communication with Amgen, Inc.). In monkeys, the half-life of FL has been shown to be 24-48 h when administered subcutaneously, and ~11 h when administered intravenously (authors' communication with Amgen, Inc.). However, detailed pharmacokinetic studies of FL in mice have not been performed. All mice underwent echocardiographic studies four days prior to coronary occlusion/reperfusion and at 48 h and 4, 8, 16, 32, and 48 wk after reperfusion. Mice were sacrificed at 48 wk after reperfusion.

Myocardial infarction

The experimental preparation has been described in detail [2,17,18]. Briefly, mice were premedicated with atropine sulfate (0.04 mg/kg i.m.), anesthetized with pentobarbital sodium (60 mg/kg i.v.), intubated, and ventilated. 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. To replace blood loss, blood from a donor mouse was given intravenously. 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 for 30 min. 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, 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 [2,17,18]. Ketoprofen (5 mg/kg s.c.) was administered for pain every 24 h postoperatively for at least 3 days or longer as necessary. Mice also received cefazolin (25 mg i.m.) daily for 3 days postoperatively.

Echocardiography

Serial echocardiograms were obtained at baseline (4 days prior to coronary occlusion) and at 48 h and 4, 8, 16, 32, and 48 wk after reperfusion. Prior to echocardiography, mice were weighed and anesthetized with isoflurane inhalation (3% for induction, 1% for maintenance). The anterior chest was shaved and the mice were placed on a heating pad in the left lateral decubitus position. A rectal temperature probe was placed to insure that the body temperature remained very close to 37.0°C during the study. LV structure and function were assessed by previously validated two-dimensional, M-mode, and Doppler echocardiographic techniques [2,18]. Echocardiographic images were obtained using a Philips HDI 5000 SonoCT ultrasound system equipped with a 12-5 MHz phased-array probe fitted with a 0.3 cm standoff and a 15-7 MHz broadband linear probe. Digital images were analyzed off-line according to modified American Society for Echocardiography standards using the ProSolv image analysis software. LV end-diastolic (LVEDD) and end-systolic diameters (LVESD) were measured from M-mode tracings obtained at the midpapillary level. LV mass was estimated from the M-mode data and LV end-diastolic volume (LVEDV) was calculated using the modified Simpson's method. Analysis of data was performed by an investigator who was blind to the treatment assigned and was unaware of data from other modalities.

Morphometry and infarct size

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 [2,22]. The right atrium was cut to allow drainage. 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 area fraction was calculated by computerized planimetry (Image-Pro Plus) of digital images of three Masson's trichrome-stained serial LV sections taken at 0.5-1.0 mm intervals along the longitudinal axis [2,22]. All morphometric analyses were performed by investigators who were blind to the treatment allocation.

Statistical analysis

Data are reported as mean $\pm$ SEM. Infarct size data were analyzed with one-way ANOVA. Serial echocardiographic parameters were measured with a two-way (time and group) ANOVA followed by Student's *t*-tests with the Bonferroni correction [23]. A *P* value less than 0.05 was considered statistically significant. All statistical analyses were performed using the SPSS software (version 8, SPSS, Inc., Chicago, IL).

Results

Exclusions

A total of 68 mice were used. Of these, 26 died within 48 h after surgery. One mouse in group I died during the 4th week of follow-up, and 6 mice were excluded from the study due to failure of the coronary occluder, leaving a total of 7, 10, 10, and 8 mice in groups I, II, III, and IV, respectively.

Myocardial infarct size

The infarct area fraction (which measures the average area of scarred tissue, expressed as a percent of the LV area in three LV sections 0.5-1.0 mm apart) did not differ significantly among the four groups (Fig. 2).

G-CSF+FL therapy attenuates LV systolic dysfunction during long-term follow-up

Before coronary occlusion (baseline), all parameters of LV function, measured by echocardiography, were similar in groups I-IV (Fig. 3). At 48 h after reperfusion, the degree of LV systolic functional impairment did not differ among the groups (Figs. 3E, F, G); indicating that the extent of injury sustained during ischemia/reperfusion was comparable. As expected, in vehicle-treated mice the infarct wall thickening decreased (Figs. 3A and E) and the LVEDD increased at 48 wk of follow-up compared with baseline (Figs. 3A and F). In mice treated with G-CSF+FL, at 48 wk thickening fraction in the infarct wall (an index of regional function) was greater (Fig. 3E) and the LVEDD smaller (Fig. 3F) compared with vehicle-treated mice. Vehicle-treated mice exhibited a progressive deterioration in LVEF between 48 h and 48 wk after reperfusion; this worsening in LVEF was attenuated in mice treated with G-CSF+FL, resulting in a markedly greater LVEF at 48 wk compared with vehicle-treated mice ($40.96 \pm 2.27\%$ vs. $23.52 \pm 3.85\%$ in groups II and I, respectively; $P < 0.05$; Fig. 3G). Consistent with our earlier observations [2], LVEF was significantly greater in G-CSF+SCF-treated mice than in vehicle-treated mice at 4 wk after reperfusion (Fig. 3G); however, at 48 wk LVEF was not significantly different between these two groups (Fig. 3G), indicating that the improvement in LVEF with G-CSF+SCF therapy is not sustained during longer follow-up. Therapy with G-CSF alone was not effective in ameliorating LV dysfunction at any time-point (Figs. 3D-G). Compared with baseline, heart rate increased in all groups during follow-up (supplemental Table 1), indicating neurohumoral activation in response to heart failure. Consistent with the amelioration of LV dysfunction, heart rate during follow-up was significantly lower in G-CSF+FL-treated mice compared with vehicle-treated mice. Compared with baseline, stroke volume decreased during follow-up in groups I and IV (supplemental Table 1). However, in G-CSF

+FL-treated mice, the initial decrease in SV was followed by a compensatory increase secondary to the increase in LVEDV, indicating improved contractility in adaptation to greater preload (supplemental Table 1). This improvement was sustained until 48 weeks.

G-CSF+FL therapy halts LV remodeling

During follow-up, LVEDD and LVEDV increased progressively in all groups, consistent with postinfarct LV remodeling (Figs. 4A-F). However, in G-CSF+FL-treated mice LVEDD and LVEDV at 48 wk were significantly smaller compared with vehicle-treated mice, indicating sustained improvement in LV remodeling with G-CSF+FL treatment (Figs. 4E and F). In contrast, G-CSF+SCF therapy failed to improve LVEDD and LVEDV. Treatment with G-CSF alone was also ineffective in improving LV remodeling during long-term follow-up (Figs. 4D-F).

G-CSF+FL therapy attenuates LV hypertrophy

Consistent with progressive LV remodeling after infarction, echocardiographically-estimated LV mass at 48 wk of follow-up was significantly greater in all groups compared with baseline (preinfarct) values (Fig. 5). In the G-CSF+FL-treated group, however, the estimated LV mass was significantly smaller compared with vehicle-treated mice (170 ± 7.9 mg vs. 207 ± 18.6 mg, respectively; $P < 0.05$) (Fig. 5), further supporting a beneficial effect of G-CSF+FL therapy on postinfarct LV remodeling.

Discussion

Evidence from animal models suggests that administration of cytokines after MI improves LV function and remodeling [1,2,24-26]. However, clinical translation of these findings is hindered by the fact that many cytokine regimens are theoretically available and it is unknown which one is more likely to be effective; further, it is unknown whether the salubrious effects of cytokines are sustained. Only a small number of studies has used cytokine combinations [1, 2,26], and the follow-up was relatively short. To date, virtually nothing is known regarding the long-term effects of cytokine therapy for infarct repair. The direct comparison of cytokine therapies performed in the current study during a long follow-up (11 months) provides several novel findings that are relevant from a therapeutic standpoint. Our results show that: i) the beneficial effects of G-CSF+FL on both regional (infarct wall thickening fraction) and global (EF) LV function after an acute MI are sustained for at least 48 weeks in mice, and thus are likely to be permanent; ii) the combination of G-CSF+FL also affords a sustained improvement in LV remodeling (limiting LV dilatation and hypertrophy); iii) in contrast, therapy with G-CSF+SCF has no significant effect on LV remodeling, and although it produces an LV functional improvement at 4 weeks, this early benefit is gradually lost over the ensuing months; and iv) G-CSF alone fails to improve LV structure and function. Taken together, these results identify distinct advantages of G-CSF+FL therapy for postinfarct cardiac repair and establish its efficacy during a long follow-up - a critical consideration for therapeutic translation.

No previous study has examined the long-term effects of hematopoietic cytokines after MI. It is known that following hematopoietic cytokine therapy, BMCs are mobilized in large numbers from the bone marrow into the peripheral blood [27,28] and home into the infarcted myocardium [1,2,29], where various chemoattractants are expressed [30]. Accordingly, BMCs are thought to play an important role in cytokine-induced cardiac repair [1,2]. However, some studies of BMC transplantation in animals as well as humans suggest that the structural and functional benefits imparted by BMC therapy on the infarcted heart are rather transient, and tend to disappear during longer follow-up [7,8], although this has not been a consistent finding [31]. Clearly, this was not the case with the G-CSF+FL combination in the present study, since the improvement in LV function and structure induced by these two cytokines persisted for at

least 48 wk after MI in mice - a duration that corresponds to many years in humans. In stark contrast to the sustained nature of this action, the effects of the G-CSF+SCF combination were ephemeral; in G-CSF+SCF-treated mice, LV function was improved at 4 wk after MI, but at later time points it was not significantly different from controls, nor was there any evidence of other structural or functional benefits. Furthermore, the significant reduction in heart rate during follow-up in G-CSF+FL-treated mice was consistent with improvement in neurohormonal activation associated with heart failure. These data reveal important, and heretofore unknown, differences in the long-term actions of various cytokine combinations.

Consistent with our earlier observations [2], therapy with G-CSF alone was not effective in improving LV function during either a short or a long follow-up. After 48 wk, LVEF in G-CSF-treated mice was similar to that in mice treated with G-CSF+SCF, and neither was significantly different from controls. This is consistent with results from several clinical trials in which G-CSF as a monotherapy failed to improve cardiac function in patients with acute MI [10,12,13]. The results of these clinical trials were verified in subsequent meta-analyses, in which G-CSF therapy in unselected patients was not associated with improved outcomes compared with control patients [15,16]. Interestingly, however, our meta-analysis [16] showed LV functional improvement in G-CSF-treated patients that had lower LVEF, suggesting that G-CSF may be efficacious in animals or patients with large infarcts and severely compromised LV function.

The mechanistic basis for the observed differences in outcomes among different cytokine combinations remains unclear. In our previous study using chimeric mice with EGFP-labeled BMCs, therapy with G-CSF+FL was associated with greater regeneration of myocytes as well as nonmyocytes compared with other treatment regimens (vehicle, G-CSF+SCF, and G-CSF alone) [2]. It is plausible that functional improvement resulting primarily from regeneration of myocytes may afford longer-lasting benefits compared with benefits resulting largely from paracrine effects. Moreover, in our previous study, we reported a differential mobilization of BMC subsets by different cytokine regimens, with G-CSF+FL mobilizing the greatest number of Lin-/Sca-1+/c-kit+ bone marrow stem cells [2]. It is therefore conceivable that myocardial homing of different BMC subpopulations may account for the differential effects of various cytokine combinations. Furthermore, cytokines are known to exert direct effects on the surviving myocytes; the long-term actions of cytokines on myocardial matrix, fibrosis, and remodeling remain poorly understood.

The mechanism underlying the observed superiority of G-CSF+FL in cardiac repair is likely multifactorial. In our previous study [2], treatment with G-CSF+FL not only mobilized greater number of primitive BMCs, but also was associated with greater number of cardiac differentiated BMCs in the myocardium. Indeed, previous studies have shown that although G-CSF and FL alone demonstrate a modest effect on mobilization and engraftment of hematopoietic stem cells, they exhibit striking synergy when used in combination [32,33]. In addition, BMC-independent direct effects of FL on cell survival and apoptosis may also contribute to the synergistic benefits. Binding of FL to its receptor activates PI3-K/Akt and RAS signaling cascades, leading to inhibition of apoptosis and increased cell proliferation, respectively [34]. The activation of RAS stimulates downstream effectors such as Raf, MAPK, ERK1/2, which activates cAMP-response element binding protein (CREB), ELK, and STATs, and leads to transcription of genes involved in cell proliferation [34]. Future studies will be necessary to identify whether G-CSF+FL therapy induces favorable changes in the myocardial matrix via the activation of these pathways.

In conclusion, our results demonstrate that therapy with G-CSF+FL initiated shortly after a reperfused MI improves LV function and remodeling and that these benefits last for at least 48 wk after MI; in the murine model, such a duration indicates that the observed salubrious

effects are likely permanent. In contrast, therapy with G-CSF+SCF, although effective in the short term (first few weeks), fails to offer sustained functional benefits. These results reveal heretofore unknown differences among cytokine regimens, and have important implications for the design of clinical trials of cytokine therapy in patients with postinfarct LV dysfunction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Experimental Protocol

Figure 1. Experimental protocol

Four groups of mice were used. On day 1, 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: group I, vehicle (days 1-10); group II, G-CSF (250 µg/kg/d, days 1-5) + FL (333 µg/kg/d, days 1-10); group III, G-CSF (250 µg/kg, days 1-5) + SCF (200 µg/kg, days 1-5); and group IV, G-CSF (250 µg/kg, days 1-5). All mice in groups I-IV underwent echocardiographic studies 4 days prior to coronary occlusion/reperfusion and at 48 h and 4, 8, 16, 32, and 48 wk after reperfusion. Mice were sacrificed at the end of 48 wk after coronary reperfusion.

Figure 2. Myocardial infarct size

Myocardial infarct area fraction assessed from Masson's trichrome-stained hearts in groups I-IV. ○, Individual mice; ●, mean±SEM.

Figure 3. Echocardiographic data

(A-D) Representative M-mode echocardiographic images from vehicle-treated (A), G-CSF +FL-treated (B), G-CSF+SCF-treated (C), and G-CSF-treated (D) mice at 48 wk 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 heart and virtually absent in the vehicle-treated heart. (E) Infarct wall thickening fraction in groups I-IV at baseline (preinfarct) and at 48 h and 48 wk after reperfusion. (F-G) Quantitative assessment of LV systolic function. Echocardiographic measurements of LV end-systolic diameter (F) and ejection fraction (G) in control and cytokine-treated mice (groups I-IV) are illustrated at baseline and at 48 h and 4, 8, 16, 32, and 48 wk after reperfusion. The administration of G-CSF+FL improved LV systolic function 48 wk after MI, while the early improvement in EF with G-CSF+SCF therapy was lost during longer follow-up. Data are mean $\pm$ SEM. n=7-10 mice/group. * $P < 0.05$ vs. group I.

Figure 4. Assessment of LV remodeling

Representative Masson's trichrome-stained myocardial sections from vehicle-treated (A), G-CSF+FL-treated (B), G-CSF+SCF-treated (C) and G-CSF-treated (D) mice. Scar tissue and

viable myocardium are identified in blue and purple, respectively. The LV cavity is smaller and the infarct wall is thicker in the G-CSF+FL-treated mouse compared with the control mouse. **(E-F)** Quantitative assessment of LV remodeling. Panels **E-F** illustrate echocardiographic measurement of LV end-diastolic diameter (**E**) and LV end-diastolic volume (**F**) at baseline and at 48 h and 4, 8, 16, 32, and 48 wk after reperfusion. The administration of G-CSF+FL improved LV remodeling at 48 wk after MI. Data are mean $\pm$ SEM. n=7-10 mice per group. * P <0.05 vs. group I.

Figure 5. Assessment of LV hypertrophy

Echocardiographically estimated LV mass at 48 wk after MI was significantly less in G-CSF +FL-treated hearts compared with vehicle-treated hearts. Data are mean $\pm$ SEM. n=7-10 mice/group. * $P < 0.05$ vs. group I.