Improvement in cardiac function after bone marrow cell therapy is associated with an increase in myocardial inflammation

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Sun J, Li S, Liu S, Wu J, Weisel RD, Zhuo Y, Yau TM, Li R, Fazel SS. Improvement in cardiac function after bone marrow cell therapy is associated with an increase in myocardial inflammation. Am J Physiol Heart Circ Physiol 296: H43–H50, 2009; doi:10.1152/ajpheart.00613.2008.—The mechanisms for the beneficial impact of bone marrow cell (BMC) therapy after myocardial infarction (MI) are ill defined. We hypothesized that the implanted cells improve function by attenuating post-MI inflammation and repair. In mice, $3 \times 10^5$ fresh BMCs were implanted immediately after coronary ligation. Cardiac function was evaluated over time. Inflammatory cytokines and cells were measured, and their impacts on the (myo)fibroblastic repair response, angiogenesis, and scar formation were determined. All differences below had $P$ values of $<0.05$.

BMC implantation reduced the decline in fractional shortening and ventricular dilation. Invasive hemodynamics confirmed a difference in systolic function at day 7 and diastolic function at day 28 favoring the BMC group. Interestingly, BMC implantation caused a 1.6-fold increase in the number of macrophages infiltrating the infarct but did not affect neutrophils. This increase was associated with a 1.9-fold higher myocardial TNF-$\alpha$ level. The heightened inflammatory response was associated with a 1.4-fold induction of transforming growth factor-$\beta$ and a 1.3-fold induction of basic fibroblast growth factor. These changes resulted in a 1.6-fold increase in $\alpha$-smooth muscle actin and a 1.9-fold increase in total discoidin domain receptor 2-expressing cells in the BMC group. These two markers are expressed by cardiac (myo)fibroblasts. Capillary density in the border zone increased 2.0-fold. Consistent with a more robust repair-mediated scar “contracture,” the final scar size was 0.7-fold smaller in the BMC group. In conclusion, after MI, BMC therapy induced a more robust inflammatory response that improved the “priming” of the (myo)fibroblast repair phase. Enhancing this response may further improve the beneficial impact of cellular therapy.

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address this hypothesis, we examined the response to intramyocardial bone marrow cell (BMC) delivery immediately after coronary ligation in a murine model.

METHODS

Animal procedures. All the experimental procedures were approved by the Animal Care committee of Toronto General Research Institute. Female C57BL/6 mice of 8–10 wk of age were obtained from Charles River Laboratories. Mice were intubated and ventilated with 2% isoflurane. Through a thoracotomy, the left anterior descending coronary artery was permanently ligated, which resulted in the infarction of ~30% of the LV. Two study groups were studied: 1) the medium group and 2) the BMC injection group. For the medium group, 15 μl of serum-free medium was injected in three injections across the infarcted myocardium immediately after ligation using a 27-gauge insulin needle. For the BMC group, 3 × 10^5 cells were suspended in 15 μl of serum-free medium and injected using an identical protocol.

Preparation of BMCs. Syngeneic BMCs were obtained by flushing the cavity of the tibia and femur of 4–6-wk-old C57BL/6 mice. After isolation, cells were lysed twice with cell lysis buffer (0.16 M NH4Cl and 0.017 M Tris; pH 7.65) and neutralized with Iscove’s modified Dulbecco’s medium (IMDM) + 10% FBS. Cells were subsequently resuspended in serum-free IMDM in preparation for cell transplantation.

Cardiac function and morphometry. Cardiac function was evaluated by echocardiography. Mice were sedated with 2% isoflurane, and echocardiographic images were recorded as previously described (5). Heart rate, LV end-diastolic diameter (LVEDD), and LV end-systolic diameter (LVESD) were measured. Fractional shortening (FS) of the LV was calculated as follows: FS = [(LVEDD – LVESD)/LVEDD] × 100.

Pressure-volume analysis was carried out in a random subset of animals on day 7 and on all surviving animals on day 28. Heparinized saline (1 ml) was injected intraperitoneally 30 min before the procedure to fluid load animals. Under positive pressure ventilation, a micromanometer and conductance 1.4-Fr catheter (Millar Instruments, Houston, TX) was introduced into the LV through the right carotid artery. Pressure-volume loops were obtained during brief apnea.

Flow cytometry analysis. For flow cytometry, hearts were collected, and the right ventricular free wall was excised. The remainder of the heart was divided into infarcted and noninfarcted segments before digestion using 0.1% collagenase type II (Worthington) at 37°C for 30 min. Cells were filtered through a 70-μm filter and resuspended in PBS supplemented with 2% FBS and 0.1% sodium azide. One million cells were taken for antibody staining of FITC-conjugated rat anti-mouse neutrophil (Serotec) or rat anti-mouse Mac-3 (BD Pharmingen) or goat anti-discoindin domain receptor 2 (DDR2; Santa Cruz Biotechnology). All antibody incubation was carried out for 30 min at 4°C in the dark. Alexa fluor 488-conjugated donkey anti-rat or anti-goat antibodies (Molecular Probes) were added for staining with mouse Mac-3 or DDR2, respectively. Isotype-identical antibodies served as controls (Becton Dickinson). Cells were analyzed using a Beckman Coulter EPICS XL flow cytometer with identical antibodies served as controls (Becton Dickinson). Cells were resuspended in PBS supplemented with 2% FBS and 0.1% sodium azide. One million cells were taken for antibody staining of FITC-conjugated rat anti-mouse neutrophil (Serotec) or rat anti-mouse Mac-3 (BD Pharmingen) or goat anti-discoindin domain receptor 2 (DDR2; Santa Cruz Biotechnology). All antibody incubation was carried out for 30 min at 4°C in the dark. Alexa fluor 488-conjugated donkey anti-rat or anti-goat antibodies (Molecular Probes) were added for staining with mouse Mac-3 or DDR2, respectively. Isotype-identical antibodies served as controls (Becton Dickinson). Cells were analyzed using a Beckman Coulter EPICS XL flow cytometer with EXP032 ADC software. The fluorescence intensity of 10,000 cells for each sample was recorded.

Quantification of myocardial cytokine levels. To determine myocardial cytokine levels, heart samples were separated into infarcted and noninfarcted segments as described above and homogenized in liquid N2. Total protein was extracted from powdered tissue in lysis buffer [20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM Na pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM PMSF] for 1 h on ice. After centrifugation at 10,000 g for 10 min, the supernatant was collected, and the protein concentration was determined using a Bio-Rad DC protein assay kit. Levels of TNF-α (Endogen), transforming growth factor (TGF)-β and basic fibroblast growth factor (bFGF; R&D Systems) were determined using ELISA following manufacturer’s instructions and were normalized to picograms per milligram of total protein.

Immunohistochemistry. To determine capillary density in the myocardium, the LV was perfused from the apex with 0.9% saline, subsequently embedded in OCT compound, and then snap frozen in liquid N2. The 5-μm-thick transverse sections were stained with rat anti-mouse CD31 (BD Pharmingen) followed by Alexa fluor 488-conjugated donkey anti-rat antibodies (Molecular Probes). 4’,6-Diamidino-2-phenylindole (Sigma) was used to stain the nucleus of the cells. The number of FITC-positive capillary structures was determined per high-power field by a Nikon fluorescent microscope in five randomly selected fields per section in a blinded fashion.

For α-SMA staining, heart samples were first perfusion fixed in 10% formalin and then sectioned. Sections were immunohistochemically stained for α-SMA (Sigma) according to the manufacturer’s protocols and counterstained with hematoxylin and eosin. Four or five fields from each of 5 sections/animal were randomly selected for analysis in a blinded fashion.

Statistical analysis. All values are presented as means ± SD. Comparison between two groups was carried out by unequal variance, the two-tailed Student’s t-test. Time-course data were analyzed by two-way ANOVA. Post hoc testing using Bonferroni’s correction was used to identify time points at which differences emerged. P < 0.05 was considered to be significant.

RESULTS

First, we performed functional analyses to determine the earliest time point at which differences between groups become evident and to characterize the nature of the difference. Serial echocardiography (sham group, n = 6; medium group, n = 9; and BMC group, n = 9) confirmed our previous observations that bone marrow mononuclear cell implantation prevents the further decline in cardiac function after the precipitous decrease observed in the first 24 h after coronary ligation (Fig. 1A). This difference became evident, as measured by FS, within 1 wk of cell implantation with no further marked difference developing over the ensuing 21 days. Measurements of chamber dimensions also showed that the major difference between the groups arose during the first week (Fig. 1B). Pressure-volume analysis (sham group, n = 6; medium group, n = 8–9; and BMC group, n = 8–9) in a separate series of mice on days 7 and 28 was then carried out (Fig. 1, C–J). This analysis confirmed and refined the differences between the groups. Whereas the impact on systolic function was clear by day 7, as measured by end-systolic pressure, maximal dP/dt (dP/dt), and ejection fraction, the impact on diastolic parameters was only fully evident at the time point of day 28. The latter measures included end-diastolic pressure and the time constant of LV isovolumic relaxation and suggested differences in myocardial stiffness. Thus, cell implantation not only had an impact on contractile unit preservation/restoration at the early time point but also on ongoing chronic LV remodeling.

Based on these results, we focused attention on the first week after BMC implantation and evaluated the inflammatory response in the injured myocardium elicited by both MI and cell therapy (n = 3 animals per time point per group). As anticipated, coronary ligation caused a robust infiltration of inflammatory cells (both neutrophils and macrophages), which peaked at day 7 after MI and returned to baseline by day 28 (Fig. 2, A and B). The implantation of BMC did not affect the binding of neutrophils and monocytes but did significantly reduce the infiltration of macrophages (Fig. 2, C and D). Addition of conventional COX-2 inhibitors to the implants at the time of BMC injection significantly reduced the infiltration of macrophages but did not influence the binding of neutrophils and monocytes (Fig. 2, E and F). Moreover, all these effects were not observed when BMCs were implanted in sham-operated animals (Fig. 2, G–L). These results suggested that BM-derived cells exert a protective effect against chronic inflammation induced by MI.

To determine whether BM-derived cells also protect against chronic myocardial fibrosis, we studied the influence of BMC therapy on interstitial collagen content. For this purpose, mice were subjected to coronary ligation 6 weeks after BMC or medium implantation and sacrificed 7 days later. To assess fibrosis, we determined the expression of α-SMA, a marker for myofibroblasts, and procollagen I (Col-I), a major component of collagen fibers. Both α-SMA and Col-I expression was increased in the BMC group compared with the medium group (Fig. 3, A and B). Thus, these results suggested that BM-derived cells can protect against chronic inflammation and fibrosis in the injured myocardium.
first wave of inflammatory cell influx into the infarcted region, and the total number of neutrophils was the same in the two groups (Fig. 2A). However, BMC implantation caused a more rapid and robust influx of Mac-3-expressing macrophages (Fig. 2B) into the infarcted region, with larger differences occurring on day 7 followed by a return to baseline values by day 28. TNF-α (a major proinflammatory cytokine) levels (n = 6 animals per time point per group) were nearly twice as high in the infarcted zone of the BMC group compared with controls. The implantation of BMCs had a marked impact not only promotes angiogenesis but arteriogenesis as well. Fibroblasts transform to myofibroblasts under TGF-β and proliferate with bFGF after being wounded. As anticipated, in our control mice, MI caused a slow increase in both cytokines (n = 6 animals per time point per group), the BMC group had nearly 400 more capillaries/mm² than the control group (Fig. 3A–C). Interestingly, the rate of apoptosis (n = 3 animals per time point per group) was also measured to be higher in the BMC group on days 1 and 3 (Fig. 3A and B).

Given our speculation that inflammation primes the reparative response (6), we next evaluated the impact of cell implantation on early and late myocardial reparative processes. We focused on angiogenesis and the less-well studied myofibroblast response. Coronary ligation caused a loss of capillaries, defined as structures expressing CD31, which were slowly regenerated up to day 28 in the border zone (Fig. 3C and D). Within 1 wk of coronary ligation (n = 5 animals per time point per group), the BMC group had nearly 400 more capillaries/mm² than the control group (Fig. 3D). Similarly, post-MI recovery of a fraction of arterioles (n = 5 animals per time point per group), defined as structures with lumens coated with α-SMA-expressing cells, in the border zone continued to day 28 in controls. The implantation of BMCs had a marked impact on the number of arterioles in the border zone that became evident by day 28 (Fig. 3, E and F). Thus, BMC implantation caused a marked upregulation of TGF-β by days 3 and 7, which normalized by day 28, in the infarcted region (Fig. 4A). BMC implantation also caused higher bFGF levels, which lasted to day 28 (Fig. 4B). Consistent with these results, the number of myofibroblasts, as detected by expression of the collagen receptor DDR2 (n = 3 animals per time point per group; Fig. 5A) and α-SMA (n = 5 animals per time point per group; Fig. 5, B and C), was increased in both control and BMC groups after coronary ligation, with the peak detected on day 7. In controls, sheets of α-SMA-expressing tissue were evident throughout the infarcted area extending from border zone to border zone. The increase in the myofibroblast response was more robust in the BMC group (Fig. 5C). To corroborate these findings, we undertook morphometric measurements of perfusion-fixed hearts performed at physiological pressures (n = 6 animals per time point per group). Whereas the scar size of control mice increased in relative size from 40.5 ± 4% to 49.0 ± 6% (P = 0.02) of the LV, the BMC

Fig. 1. Analysis of cardiac function after cell transplantation. A and B: results of echocardiography showing more rapid loss of function and ventricular dilation in the control group (sham group, n = 6; medium group, n = 9; and bone marrow cell-transplanted (BMC) group, n = 9). These measurements were performed at similar heart rates. LVEDD, left ventricular end-diastolic dimension; MI, myocardial infarction. *P < 0.05, medium group compared with BMC group. C–J: results of pressure-volume analysis at two time points (days 7 and 28) showing early difference in systolic function and a later-manifested difference in diastolic function in favor of the BMC group. Heart rates for the medium group were 290 ± 108 beats/min on day 7 and 182 ± 56 beats/min on day 28, and heart rates for the BMC group were 329 ± 86 beats/min on day 7 and 240 ± 85 beats/min on day 28 (sham group, n = 6; medium group; n = 8–9; and BMC group, n = 8–9). Tau, time constant of isovolumic relaxation.*P < 0.05, medium group compared with BMC group.
group had a relatively more stable scar over the same period starting at $30.0 \pm 4.2\%$ and ending at $34.8 \pm 4.4\%$ ($P = 0.08$) of the LV (Fig. 5D), as would be anticipated from an enhanced myofibroblast response.

**DISCUSSION**

We have previously documented, using a homogenous preparation of bone marrow mesenchymal stem cells, that direct myocardial cell implantation improves cardiac function in the absence of detectable cardiomyogenic differentiation of the implanted cells (21). These findings suggested that the impact of the cells on cardiac function is largely independent of cardiomyocyte differentiation. The findings reported from multiple groups in both animal models and human clinical trials, which used different donor cell sources, have resulted in a similar degree of functional benefit (15). Again, this observation suggests that the impact of the cells might be independent of cell type to some extent and, more importantly, that the impact of the cells on cardiac function are indirect, possibly through paracrine or other mechanisms.

Within this context, we examined the hypothesis that cell implantation directly into the myocardium immediately after coronary ligation affects the inflammatory response to MI. We noted several interesting observations. First, the impact of cell implantation is apparent very early after transplantation, within the inflammatory phase of repair. Second, both inflammatory cellular and cytokine responses were modulated by the implanted cells. Third, the impact was not a generalized up- or downregulation; instead, whereas the neutrophilic influx was unchanged, implanted cells caused a more robust macrophage response that only became apparent between days 3 and 7 after implantation. Finally, as we expected, this impact on the inflammatory response was followed by an improved proliferative “repair” response in terms of both angiogenesis and myofibroblast-induced stabilization of the size of the infarct scar.

Fig. 2. Cell transplantation attenuates the inflammatory response to MI. A: cell implantation did not affect the magnitude of the neutrophilic response ($n = 3$ animals/time point). B: cell implantation, however, increased the number of macrophages that infiltrated the infarcted area ($n = 3$ animals/time point). C: effect of the cells on myocardial TNF-α was pronounced, with a 2-fold increase in TNF-α levels that became obvious at day 7 ($n = 6$ animals/time point). *$P < 0.05$, medium group compared with BMC group.
One of the roles of the macrophage lineage is to clear necrotic debris. Likely, one of the main reasons why treatment of patients with acute MI with corticosteroids caused poor cardiac repair was the delayed and aberrant clearing of dead cardiomyocytes (15). Another increasingly recognized role of macrophage infiltration is the elaboration of various molecules that appear to be critical in the propagation of wound repair processes. Interference with macrophage chemotaxis to the infarcted myocardium by ablation of the monocyte chemoattractant protein-1 gene leads to a reduction in total myocardial TGF-β2 and IL-1β (4), both of which are critical for the differentiation of fibroblasts to myofibroblasts that express α-SMA contractile elements and are important for wound contracture (25) and, as we speculate, also for infarct scar stabilization. In addition, macrophages mediate a differential response depending on whether they engulf necrotic or apoptotic cells, which may underlie some of the differential cytokine expression patterns in the peri-infarct border zone versus the core of the infarct. For instance, hepatocyte factors and VEGFs are increased in macrophages upon phagocytosis of apoptotic cells but decreased upon phagocytosis of necrotic cells (9). Given that a significant number of cells die by apoptosis in the border zone, it is plausible that macrophage-derived growth factors account for the impressive angiogenesis seen with BMC therapy in the border zone. Although the overlapping and diverse functions of macrophages in tissue repair and regeneration are less well characterized compared with their role in innate immunity, it is becoming clear that they play an important regulatory role in this process (22).

Within this context, we believe that the impact of the cells on macrophage influx is a biologically important observation and not purely an epiphenomenon.

After MI, TGF-β plays a role in the resolution of the inflammatory response, and elevated levels of TGF-β in the early phase are important to preserve cardiac function (2, 13). However, prolonged elevations of the TGF-β signal can result in excessive fibrosis, in particular in the remote myocardium. In our model, levels of this cytokine were markedly higher in early time points but normalized by day 28. Although we did not specifically evaluate fibrosis in the two groups, our cardiac function measurements suggest diastolic dysfunction in the control group with elevated end-diastolic pressures. The role of the cytokine bFGF after MI is more straightforward. This cytokine promotes endothelial and fibroblast proliferation and aids in scar stabilization. Overexpression of this cytokine enhanced its beneficial impact, suggesting no obvious ceiling effect (26). The elevated levels of bFGF in our model, which lasted to the end of the study, as well as the smaller infarct size in the BMC group, strongly supports the previous data.

The mechanism by which BMC influences post-MI inflammation is unclear. Given that the neutrophilic response was not augmented, it is unlikely that the heightened macrophage response is driven by a higher dead cell burden after the death of a portion of the implanted cells. In this context, it is important to note that the death of the implanted cells by apoptosis has been previously postulated to attenuate the inflammatory response (24, 28). This observation goes hand in hand with the observed higher rate of apoptosis in the BMC.
We have previously shown that implantation of bone marrow mesenchymal stem cells causes a more robust release of the endothelial progenitor cell population within the bloodstream (5). We speculated that this was caused by elaboration of mobilizing cytokines by the implanted cells, and, indeed, systemic levels of stem cell factor, which we have subsequently shown to be necessary for endothelial progenitor cell mobilization (8), were markedly upregulated in the BMC group. Although we do not have direct results from the present series of experiments, we believe that the paracrine influence of implanted cells drove, to a large extent, the modulation of the inflammatory response that we observed.

The increase in TNF-α levels that we observed was unexpected. Certainly, the long-term overexpression of TNF-α results in cardiomyocyte dysfunction and death (12). In addition, mice that are Tnfα-null are protected after coronary ligation compared with wild-type controls (23). Although these data suggest a detrimental role for TNF-α, clinical blockade of TNF-α has not improved patient outcome in patients with established heart failure (1, 3). Furthermore, we have shown that TNF-α is a critical inducer of stem cell factor expression by endothelial cells (8), and the higher TNF-α levels we observed in our study could have resulted in the elaboration of cardioprotective and angiogenic cytokines. Also, the transient expression of TNF-α caused by cell implantation might have reduced the adverse effects of this cytokine. Finally, the spatial restriction of TNF-α to the infarct zone could have prevented its detrimental effects in the remote region of the myocardium.

Functional experiments have suggested that the impact of the cells is largely on the systolic function in the early phase followed by a gradual impact on the diastolic function. Although our notion that the implanted cells improved the myofibroblast response and scar stabilization supports the observation that the treatment group had smaller infarcts, we are unsure if it fully accounts for the very early difference in systolic function. Given our belief that widespread cardiomyo-
genic differentiation and incorporation into the cardiac syncitium by a heterogeneous bone marrow mononuclear cell population is unlikely, we speculate that the cells limited cardiomyocyte loss within the border zone. The results of our morphometric analysis of scar size support this notion. Also, the increase in capillary formation observed within the first 7 days in the BMC group could have contributed to the improved cardiac function by both preserving cardiomyocytes and reducing the stunned myocardium.

The present study has several important limitations. Our observations are based on direct myocardial implantation of cells immediately after coronary ligation. This is unlikely to develop into a clinical strategy to improve post-MI care. Although we have suggested a role for macrophages in enabling a more efficient repair response, we do not have causal proof that this is the case. We did not elaborate the mechanism by which cell implantation modulates the inflammatory response. The functional benefit seen purely by modulating the inflammatory response may be short lived, as has been shown in clinical trials as well (18). It is also possible that what we observe are simply epiphenomena that are not related to the real mechanism by which cell implantation improves cardiac function.

The limitations notwithstanding, these results raise the possibility that some of the beneficial impact of cell implantation could very well be mediated by nonspecific mechanisms such as effects on inflammation. Two corollaries follow from this conclusion. First, the restriction on specific cell sources, including the requirement that the cells be autologous, could be lifted if more tailored approaches fail, given that the impact of the cells are on more general processes that do not require long-term survival of the implanted cells beyond the early infarct period. Second, new biointerventions that have more direct and specific impact should be sought. This could involve cardiomyogenic-restricted embryonic stem cells to increase contractile units or gene-enhanced cell transplantation designed to override repair responses such as increased progenitor and repair cell traffic to the heart. Clear mechanistic work needs to provide the infrastructure on which the above therapies can be based.

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