Myocardial neovascularization by bone marrow angioblasts results in cardiomyocyte regeneration

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Schuster, M. D., A. A. Kocher, T. Seki, T. P. Martens, G. Xiang, S. Homma, and S. Itescu. Myocardial neovascularization by bone marrow angioblasts results in cardiomyocyte regeneration. Am J Physiol Heart Circ Physiol 287: H525–H532, 2004; 10.1152/ajpheart.00058.2004.—The primary cardiac response to ischemic insult is cardiomyocyte hypertrophy, which initiates a genetic program culminating in apoptotic myocyte loss, progressive collagen replacement, and heart failure, a process termed cardiac remodeling. Although a few cardiomyocytes at the peri-infarct region can proliferate and regenerate after injury, no approaches are known to effectively induce endogenous cardiomyocytes to enter the cell cycle. We recently isolated, in human adult bone marrow, endothelial progenitor cells, or angioblasts, that migrate to ischemic myocardium, where they induce neovascularization and prevent myocardial remodeling. Here we show that increasing the number of angioblasts trafficking to the infarct zone results in dose-dependent neovascularization with development of progressively larger-sized capillaries. This results in sustained improvement in cardiac function by mechanisms involving protection against apoptosis and, strikingly, induction of proliferation/regeneration of endogenous cardiomyocytes. Our results suggest that agents that increase myocardial homing of bone marrow angioblasts could effectively induce endogenous cardiomyocytes to enter the cell cycle and improve functional cardiac recovery.

stem cells; myocardial remodeling; myocardial infarction

AN INTEGRAL COMPONENT of the healing process after a myocardial infarction is compensatory hypertrophy of viable cardiomyocytes at the peri-infarct rim to increase pump function in response to the loss of infarcted tissue (9, 24). However, cardiomyocyte hypertrophy initiates a genetic program that culminates in apoptotic loss of the cardiomyocytes, expansion of the initial infarct area, progressive collagen replacement, and heart failure (1, 5, 19, 20), a process termed cardiac remodeling. Pharmacological means to prevent cardiomyocyte hypertrophy remain the mainstay of postinfarct therapy to prevent remodeling and heart failure. Recent observations have suggested that a second compensatory response of viable cardiomyocytes at the peri-infarct region is to proliferate and regenerate after injury (3, 7). Although cardiomyocyte regeneration can be accomplished by providing exogenous precursors, for example, from the bone marrow (17), no approaches are known to effectively induce endogenous cardiomyocytes to enter the cell cycle.

We recently put forward the hypothesis that hypertrophied cardiomyocytes undergo apoptosis, because the endogenous capillary network cannot provide the compensatory increase in perfusion required for cell survival (12). Vascular network formation is the result of a complex process that begins in the prenatal period with induction of vasculogenesis by hemangioblasts, cells derived from the human ventral aorta that give rise to endothelial and hematopoietic elements (4, 6, 10, 13). Postnatal vasculogenesis occurs via pathways dependent on elements in the adult bone marrow and has been described in various animal models (2, 8, 15, 23, 25). In previous studies, we showed that human adult bone marrow contains cells with phenotypic and functional characteristics of embryonic angioblasts that are capable of homing to ischemic myocardium and inducing myocardial neovascularization (17). This process subsequently results in reduced cardiomyocyte apoptosis, prevention of adverse remodeling after acute infarction, and functional cardiac recovery (12).

The extent to which inductive cues from this angioblast population might additionally result in cycling and regeneration of endogenous cardiomyocytes has not been previously studied. In this study, we examined whether there was a dose-dependent relation between angioblast migration to the ischemic heart and subsequent myocardial neovascularization. Our results indicate that myocardial neovascularization results in regeneration and cell cycling of endogenous cardiomyocytes and suggest that agents that increase myocardial homing of bone marrow angioblasts could effectively induce endogenous cardiomyocytes to enter the cell cycle and improve functional cardiac recovery.

MATERIALS AND METHODS

Purification and characterization of cytokine-mobilized human CD34+ and CD34− cells. Single-donor leukopheresis products were obtained from humans treated with recombinant granulocyte colony-stimulating factor (G-CSF, 10 μg/kg sc; Amgen) daily for 4 days. Donors were healthy individuals undergoing standard institutional procedures of bone marrow mobilization, harvesting, and isolation for allogeneic stem cell transplants. Mononuclear cells were separated by Ficoll-Hypaque, and highly purified CD34+ cells (>98% positive) were obtained using magnetic beads coated with anti-CD34 MAb (Miltenyi Biotech). Purified CD34 cells were stained with fluorescein-conjugated MAbs against CD34 and CD117 (Becton-Dickinson), AC133 (Miltenyi Biotech), CD54 (ImmunoTech), CD62E (BioSource), VEGFR-2, Tie-2, von Willebrand factor, endothelial nitric oxide synthase, and CXCR4 (all obtained from Santa Cruz Biotechnology) and analyzed by four-parameter fluorescence using FACScan.

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NEOVASCULARIZATION BY BONE MARROW ANGIOBLASTS

(Becton-Dickinson). Cells positively selected for CD34 expression were also stained with phycoerythrin-conjugated anti-CD117 MAb (Becton-Dickinson) and sorted for bright and dim fluorescence using a Facsstar Plus (Becton-Dickinson) and a phycoerythrin filter. Intra-cellular staining for GATA-2 was performed by permeabilizing 1 × 10^6 cells from each of the brightly and dimly fluorescent cell populations with a Pharmingen Cytofix/Cytoperm kit by incubation for 30 min on ice with 10 μl of fluorochrome-conjugated MAb's against CD117 and CD34 surface antigens (Becton-Dickinson). After resuspension in 250 μl of Cytofix/Cytoperm solution for 20 min at 4°C, cells were incubated with a fluorochrome-labeled MAb against GATA-2 (Santa Cruz Biotechnology) or IgG control for 30 min at 4°C and analyzed by three-parameter flow cytometry. On the basis of criteria of simultaneous CD117 bright fluorescence and intracellular GATA-2 expression, ~6–12% of the CD34 + population were angioblasts.

Animals, surgical procedures, injection of human cells, and quantitation of cellular migration into tissues. Rowett (rnu/rnu) athymic nude rats (Harlan Sprague Dawley, Indianapolis, IN) were used in studies approved by the Columbia University Institute for Animal Care and Use Committee. After anesthesia, a left thoracotomy was performed, the pericardium was opened, and the left anterior descending coronary artery (LAD) was ligated. A similar surgical procedure was performed on sham-operated rats (serum was placed around the coronary artery). For studies on neovascularization and effects on myocardial viability and function, rats were also stained with phycoerythrin-conjugated anti-CD117 MAb against GATA-2 (Santa Cruz Biotechnology) or IgG control for 30 min at 4°C and analyzed by three-parameter flow cytometry. On the basis of criteria of simultaneous CD117 bright fluorescence and intracellular GATA-2 expression, ~6–12% of the CD34 + population were angioblasts.

In labeling of bone marrow-derived CD34 + and CD34 - progenitors. G-CSF-mobilized cells were immunoselected for CD34 + expression and resuspended in medium containing 20 μCi of 111In 8-Oxyquinoline (oxine) per 10^6 cells. After the cells were washed, 2 × 10^6 111In 8-Oxyquinoline (oxine)-labeled CD34 + cells obtained from a single donor after G-CSF mobilization were injected into the rat tail vein 48 h after LAD ligation. Each group consisted of 6–10 rats. We excluded all animals where the initial infarct was not large enough to cause reduction in ejection fraction by ≥50% within the first 48 h relative to normal animals. At that point, all animals included in the study were randomized, and the surgeons and other technical staff were fully blinded to the experimental conditions. Histological and functional studies were performed at 2 and 15 wk.

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Histology and measurement of infarct size. After excision at 2 and 15 wk, left ventricles from each experimental animal were sliced at 10–15 transverse sections from apex to base. Representative sections were fixed in formalin and stained for routine histology (hematoxylin and eosin) to determine cellularity of the myocardium, expressed as cell number per high-power field (HPF, ×600). Masson’s trichrome stain, which labels collagen blue and myocardium red, was used to evaluate collagen content on a semiquantitative scale (0 to 3 +) as follows: light blue (1 +), light blue and patches of dark blue (2 +), and dark blue (3 +) staining. This enabled us to measure the size of the myocardial scar using a digital image analyzer. The lengths of the infarcted surfaces, involving epicardial and endocardial regions, were measured with a planimeter digital image analyzer and expressed as a percentage of the total ventricular circumference. Final infarct size was calculated as the average of all slices from each heart. All studies were performed by a blinded pathologist. Infarct size was expressed as percentage of total left ventricular area. Final infarct size was calculated as the average of all slices from each heart.

quantitation of cardiomyocyte proliferation. Cardiomyocyte DNA synthesis and cell cycling were determined by dual staining of rat myocardial tissue sections obtained from LAD-ligated rats at 2 wk after injection of saline or CD34 + human cells and from healthy rats as negative controls for cardiomyocyte-specific troponin I and human or rat-specific Ki-67. Briefly, paraffin-embedded sections were micro- waved in a 0.1 M EDTA buffer and stained with a polyclonal rabbit antibody with specificity against rat, but not human, Ki-67 (18) at 1:3,000 dilution (gift of Giorgio Pizzuti, Columbus Laboratories, CA) or mouse monoclonal antibody recognizing human and rat Ki-67 (MB-1) at 1:300 dilution (Dako) and incubated overnight at 4°C. After they were washed, the sections were incubated with a species-
specific secondary antibody conjugated with alkaline phosphatase at 1:200 dilution (Vector Laboratories) for 30 min, and positively staining nuclei were visualized as blue with a 5-bromo-4-chloro-3-indolylphosphate-p-toluidine-nitro blue tetrazolium substrate kit (Dako). Sections were then incubated overnight at 4°C with a monoclonal antibody against cardiomyocyte-specific troponin I (Accurate Chemicals), and positively staining cells were visualized as brown through the avidin-biotin system described above. Cardiomyocytes

![Graph](image)

**Fig. 1.** Degree of neovascularization and level of protection against cardiomyocyte apoptosis are dependent on absolute angioblast numbers in ischemic heart. A and B: 1.7-fold higher numbers of medium (3–6 contiguous endothelial lining cells) and 3.3-fold higher numbers of large (>6 contiguous endothelial lining cells) capillaries in the group receiving $2 \times 10^6$ CD34+ human cells than in other groups ($P < 0.01$). Values are means ± SE of 3 separate experiments. C and D: significantly reduced numbers of apoptotic myocytes at the peri-infarct region in the group receiving $2 \times 10^6$ CD34+ human cells (C) and greater mean diameter of viable myocytes at this site (D) than in the other groups ($P < 0.01$). Values are means ± SE of 3 separate experiments. *$P < 0.05$; **$P < 0.05$. 

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progressing through the cell cycle in the infarct zone, peri-infarct region, and area distal to the infarct were calculated as the proportion of troponin I-positive cells per HPF coexpressing Ki-67. For confocal microscopy, fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG was used as secondary antibody to detect Ki-67 in nuclei. A Cy5-conjugated mouse MAb against α-sarcnomic actin (clone SC5; Sigma) was used to detect cardiomyocytes, and propidium iodide was used to identify all nuclei. In separate experiments, animals receiving saline or CD34+ cells after LAD ligation were given bromodeoxyuridine (BrdU) ad libitum in their drinking water daily. After the animals were killed, paraffin-embedded tissue was incubated with a mouse anti-BrdU antibody (Roche Molecular Biochemicals) and then with a biotinylated rabbit anti-mouse IgG antibody (Jackson ImmunoResearch) diluted 1:3,000 with D-PBS. The biotin was detected by using an avidin-biotin complex kit (Vector Laboratories), as described above.

Analyses of myocardial function. Echocardiographic studies were performed using a high-frequency linear array transducer (SONOS 5500, Hewlett Packard, Andover, MA). Two-dimensional images were obtained at midpapillary and apical levels. End-diastolic and end-systolic left ventricular volumes (EDV and ESV) were obtained by a biplane area-length method, and percent left ventricular ejection fraction (LVEF) was calculated as follows: [(EDV − ESV)/EDV] × 100.

RESULTS

Degree of neovascularization and level of protection against cardiomyocyte apoptosis are dependent on absolute angioblast numbers in the ischemic heart. We first examined the relation between the number of angioblasts injected and the resultant myocardial neovascularization. At 2 days after LAD ligation, animals were intravenously injected with saline or 10^4, 10^5, or 2 × 10^6 G-CSF-mobilized human CD34+ bone marrow cells. The migratory patterns of human CD34+ cells injected into LAD-ligated nude mice were measured by 111In labeling of CD34+ cells. We were able to determine that, after LAD ligation, 23% of the intravenously injected human cells were present in the heart 24 h after injection. In contrast, only 7% of CD34+ cells homed to the lung, 15.6% to the liver, 23.5% to the spleen. This confirms selective trafficking to the heart after LAD ligation. Induction of neovascularization at 2 wk was measured by quantitative analysis of medium- and large-sized capillaries, defined, respectively, as having three to six or more than six contiguous endothelial lining cells. Mean lumen diameter was 0.020 ± 0.002 and 0.053 ± 0.004 mm for medium- and large-sized capillaries, respectively (P < 0.001).

The group receiving 2 × 10^6 CD34+ cells demonstrated 1.7-fold more medium-sized capillaries and 3.3-fold more large-sized capillaries than the other two groups (Fig. 1, A and B; both P < 0.01). Moreover, the group receiving 2 × 10^6 CD34+ cells demonstrated significantly reduced numbers of apoptotic cardiomyocytes at the peri-infarct region and greater mean diameter of viable cardiomyocytes at this site than the other groups (Fig. 1, C and D; both P < 0.001). Together, these data indicate that the degree of myocardial neovascularization and the subsequent protection of hypertrophic cardiomyocytes against apoptosis depend on the number of angioblasts in the ischemic myocardium.

Myocardial neovascularization is accompanied by sustained regeneration of endogenous cardiomyocytes. Although myocyte hypertrophy and increase in nuclear ploidy have generally been considered the primary mammalian cardiac responses to ischemia, damage, and overload (9, 24), recent observations have suggested that human cardiomyocytes have the capacity to proliferate and regenerate in response to injury (3, 7). Therefore, we investigated whether induction of neovascularization could also result in cardiomyocyte proliferation and/or regeneration. At 2 wk after LAD ligation, rats receiving 2 × 10^6 CD34+ human cells demonstrated numerous “fingers” of cardiomyocytes of rat origin, as determined by expression of rat MHC class I molecules extending from the peri-infarct region into the infarct zone. Similar extensions were seen less frequently in animals receiving 10^6 CD34+ human cells and very rarely in the other groups. The islands of cardiomyocytes at the peri-infarct rim in animals receiving 2 × 10^6 CD34+ human cells contained a high frequency of rat myocytes with DNA activity, as determined by dual staining with a MAb reactive against cardiomyocyte-specific troponin I and a rabbit polyclonal antiserum with specificity for rat, but not human, Ki-67 (Fig. 2A) (22). Triple immunofluorescence using confocal microscopy confirmed the presence of cycling rat cardiomyocytes and demonstrated a speckled pattern of Ki-67 reactivity within cycling nuclei (Fig. 2B). In contrast, in animals receiving saline, there was a high frequency of cells with fibroblast morphology and reactivity with rat Ki-67, but not troponin I, within the infarct zone. The majority of cells staining positive for Ki-67 and troponin I were not in the process of cytokinesis, as defined morphologically. However, we did, on occasion, observe rare cardiomyocytes undergoing cytokinesis, and only in the setting of angioblast-induced neovascularization.

The number of cardiomyocytes progressing through the cell cycle was 40-fold higher at the peri-infarct region of rats receiving 2 × 10^6 human CD34+ cells than at sites distal to the infarct, where myocyte DNA activity was not different from that in sham-operated rats. The number of cycling cardiomyocytes was 20-fold higher at the peri-infarct rim of animals receiving 2 × 10^6 human CD34+ cells than in noninfarcted hearts (1.19 ± 0.2% vs. 0.06 ± 0.03%, P < 0.01) and 3.5-fold...
higher than in the same region in LAD-ligated controls receiving saline (1.19 ± 0.2% vs. 0.34 ± 0.1%, P < 0.01; Fig. 2C). Induction of cell cycling correlated with increasing concentrations of CD34⁺ cells (Fig. 2D), suggesting a dependency on a minimum number of cells necessary to overcome the threshold preventing progression of cardiomyocyte cell cycling. To better analyze differences in cell cycling throughout the experimental period, dual staining was performed for troponin I and BrdU in animals that had received BrdU daily in their drinking water from the time of surgery to death 2 wk later. Results were strikingly similar to those obtained with Ki-67 staining, and differences between the groups were even more evident, with 24-fold more cycling cardiomyocytes at the peri-infarct rim in the group receiving $2 \times 10^6$ human CD34⁺ cells than in...
noninfarcted hearts and 5-fold more than in LAD-ligated controls receiving saline (both \( P < 0.01 \)).

Enhanced cardiomyocyte survival and regeneration result in reduced fibrosis and improvement in cardiac function. We next examined the effect of increasing the number of human angioblasts trafficking to ischemic myocardium on long-term myocardial function, defined as the degree of improvement in LVEF and reduction in left ventricular end-systolic area at 15 wk after intravenous injection (Fig. 3, A and B). Only modest improvement in these parameters was observed in the group receiving \( 10^4 \) or \( 10^6 \) human CD34+ cells compared with rats receiving saline. In contrast, rats receiving \( 2 \times 10^6 \) human CD34+ cells had a mean recovery in LVEF of \( 34 \pm 4\% \) and a mean reduction in left ventricular end-systolic area of \( 37 \pm 6\% \) (both \( P < 0.001 \)). Quantitation of the ratio of fibrous tissue to myocytes at 15 wk demonstrated significantly reduced propor-

![Graph A and B showing the relation between the number of CD34+ bone marrow cells and improvement in myocardial function at 15 wk.](http://ajpheart.physiology.org/)

Fig. 3. Enhanced cardiomyocyte survival and regeneration result in reduced fibrosis and improvement in cardiac function. A and B: relation between the number of CD34+ human cells injected intravenously (\( 10^4 \), \( 10^6 \), and \( 2 \times 10^6 \)) and improvement in myocardial function at 15 wk, defined as mean left ventricular ejection fraction and mean left ventricular area at end systole. No significant improvement in these parameters was observed in groups receiving \( 10^4 \) or \( 10^6 \) human CD34+ cells compared with rats receiving saline alone. Rats receiving \( 2 \times 10^6 \) CD34+ human cells demonstrated significant recovery in left ventricular ejection fraction and reduction in left ventricular end-systolic area (both \( P < 0.001 \)). C: at 15 wk the mean proportion of scar per normal left ventricular myocardium in rats receiving \( 2 \times 10^6 \) human CD34+ cells was significantly reduced compared with rats in other groups (\( P < 0.01 \)). Values are means ± SE of 3 separate experiments. D: sections of rat hearts stained with Mason's trichrome at 15 wk after LAD ligation demonstrate greater loss of anterior wall mass, collagen deposition (blue), and septal hypertrophy in rats receiving \( 10^4 \) CD34+ human cells than in rats receiving \( 2 \times 10^6 \) CD34+ human cells.

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tions of scar per normal left ventricular myocardium in the group receiving $2 \times 10^6$ human CD34$^+$ cells compared with each of the other groups ($P < 0.01$; Fig. 3C). The overall effects of medium- and large-sized capillaries combining to protect against myocyte apoptosis and induce myocyte proliferation/regeneration are shown dramatically in Fig. 3D, where, in contrast to controls, injection with $2 \times 10^6$ human CD34$^+$ cells resulted in almost complete salvage of the anterior myocardium, normal septal size, and minimal collagen deposition.

**DISCUSSION**

Our data show that the degree of bone marrow-dependent neovascularization induced in the ischemic myocardium is directly related to the numbers of CD34$^+$ angioblasts homing to the ischemic site. As we previously showed (12), the newly developed vessels are of human and rat origin, indicating vasculosugogenesis from direct vascular incorporation and angiogenesis due to paracrine effects of secreted proangiogenic factors (11). Increasing the numbers of mobilized angioblasts in the ischemic myocardium results in development of capillaries of intermediate and large lumen size and contributes to improved heart function by two complementary mechanisms: 1) protection of at-risk, but otherwise viable, myocytes against apoptosis and 2) induction of myocyte proliferation/regeneration. Together, the results indicate that strategies to increase myocardial homing of human bone marrow-derived angioblasts may augment neovascularization of ischemic myocardial tissue and functional cardiac recovery after acute infarction.

The most striking finding in this study is that, in parallel with growth of larger-sized capillaries accompanying injection of high concentrations of human angioblasts, ischemic rat hearts developed prominent islands of regenerating myocytes at the peri-infarct region, a site recently reported to have an intrinsic capacity for self-renewal after ischemia (3, 7). In animals receiving human angioblasts, rat cardiomyocytes at the peri-infarct region demonstrated a 39-fold increase in mitotic activity compared with sites distal from the infarct and a 4.4-fold increase in the infarct region demonstrated a 39-fold increase in mitotic activity compared with sites distal from the infarct and a 4.4-fold higher mitotic activity than in saline-treated animals. The regenerative response identified here bears striking similarity to the spontaneous myocardial regeneration seen in MRL mice after cryogenic injury to the heart (14). This mouse strain demonstrates prominent spontaneous neovascularization and wound repair after myocardial or other injury (14) and has mitotic indexes approaching 20% at the site of cardiomyocyte regeneration.

Efficient delivery of nutrients and growth factors to rat cardiomyocytes by the neovascularure would provide a unifying mechanism to account for the effects on cardiomyocyte apoptosis and cardiomyocyte cycling/regeneration. Protection of hypertrophied cells against apoptosis only requires sufficient extracellular concentrations of glucose necessary to sustain glycolysis (26). In contrast, cell cycle initiation and cellular proliferation require insulin- and Akt-dependent glucose transport and phosphorylation events (21). Because Akt phosphorylation has been shown to be critical for survival of mesenchymal stem cell-derived cardiomyocytes and subsequent functional cardiac recovery (16), this may also be an important pathway involved in cycling of endogenous cardiomyocytes. Irrespective of the precise underlying mechanisms, our study demonstrates that, by inducing processes of antiapoptosis and proregeneration of endogenous cardiomyocyte tissue, significant long-term improvement in cardiac function and salvage of myocardial mass can be achieved. Strategies to optimize the number of angioblasts homing to the ischemic heart may directly impact clinical protocols using bone marrow in patients with myocardial infarction.

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