Bone marrow cells have a potent anti-ischemic effect against myocardial cell death in humans

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Objective: We sought to elucidate whether bone marrow cells ameliorate the outcomes of myocardial ischemia by reduction of cell death and to investigate whether the benefit is mediated by activation of intracellular kinases.

Methods: Muscles from the right atrial appendage of patients were subjected to 90 minutes of normothermic simulated ischemia followed by 120 minutes of reoxygenation. Bone marrow cells from the same patients were co-incubated (10^5 cells per milligram of tissue) with the muscles during the entire experimental period. Some groups were treated with the protein kinase C inhibitor chelerythrine ($10 \mu\text{mol/L}$) or the p38 mitogen-activated protein kinase inhibitor SB203580 ($10 \mu\text{mol/L}$). Creatine kinase released into the media during the reoxygenation period was measured (international units per milligram of wet tissue), cell death by necrosis was assessed by propidium iodide, and cell death by apoptosis was assessed by deoxyuride-5'-triphosphate biotin nick end labeling (percentage of aerobic control values).

Results: Creatine kinase release was significantly reduced (from 1.30 IU/mg wet tissue ± 0.11 to 0.33 IU/mg wet tissue ± 0.06 ; $P < .05$), and cell death by necrosis and apoptosis was abolished by bone marrow cells (from $30.1\% \pm 7.3\%$ and $28.1\% \pm 3.9\%$ to $-5.6\% \pm 5.1\%$ and $3.7\% \pm 5.0\%$, respectively; $P < .05$), an effect that was reversed by chelerythrine ($13.4\% \pm 4.4\%$ and $24.6\% \pm 8.2\%$, respectively) and by SB203580 ($20.1\% \pm 2.4\%$ and $19.5\% \pm 5.7\%$, respectively).

Conclusions: Bone marrow cells have a potent effect against cell death of the human myocardium in the acute phase of ischemia that may explain, at least in part, the improvement in cardiac function and the reduction in infarct size seen when bone marrow cells are injected after a myocardial infarction. These findings may have important clinical implications to optimize cell therapy with bone marrow cells. In addition, the identification that the anti-ischemic effect of bone marrow cells is mediated by the kinases protein kinase C and p38 mitogen-activated protein kinase is also clinically relevant; it suggests that some of the beneficial effect of bone marrow cells can be obtained by the activation of intracellular signaling molecules, without the need for cell injection.

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I schemic heart disease accounts for more than 50% of the reported cases of heart failure,¹ which is the leading cause of morbidity and mortality in the Western world, affecting 4.8 million people in the United States² and at least 10 million people in countries represented by the European Society of Cardiology.³ Therapies

Abbreviations and Acronyms

BMC	= bone marrow cell
MAPK	= mitogen-activated protein kinase
PKC	= protein kinase C
SI/R	= simulated ischemia followed by reoxygenation

such as thrombolysis and coronary angioplasty have improved the clinical outcomes after an acute myocardial infarction; however, the incidence of heart failure continues to increase.⁴ With the exception of heart transplantation, there is no effective treatment for heart failure, and therefore there is a need for effective new therapeutic interventions. Recently, it has been suggested that repair/regeneration of heart muscle by autologous adult stem cells may be an effective way of reversing heart failure. Bone marrow cells (BMCs) are readily available from the patient's own bone marrow, and they are inexpensive and easily prepared. Furthermore, they do not need to be expanded in culture, and their application does not require additional health resources. Because of this, BMCs can be a very attractive pool of cells for cardiac repair, and after the initial demonstration of safety use,⁵⁻⁹ several randomized clinical studies are under way.

Animal and clinical studies have reported that the intracoronary or intramyocardial injection of BMCs after a myocardial infarction improves the recovery of cardiac function and reduces infarct size.⁵⁻¹² However, whether this effect occurs by differentiation of the BMCs into vessels and muscle has been questioned.^{13,14} The observation that BMCs can spontaneously fuse with other cells and subsequently adopt the phenotype of the recipient cells^{15,16} could be an alternative mechanism of tissue repair, but this phenomenon may occur at too low a rate to be meaningful¹⁷ and has been disputed by some investigators.¹⁸ Recently, we^{19,20} and others^{21,22} have reported that progenitor or stem cells may exist within the heart, and yet another potential explanation for the beneficial effect of BMCs on the heart could be that they promote the proliferation of resident putative stem cells. Other investigators have also shown that BMCs decrease the expression of proapoptotic proteins and reduce apoptosis,²³⁻²⁵ although it is unclear from those studies whether the antiapoptotic effect is the result of increased angiogenesis and a better blood supply to the ischemic areas or is due to a direct effect of BMCs by triggering intrinsic cardioprotective mechanisms. In this connection, it has been shown that BMCs segregate growth factors and cytokines²⁶ that in turn can activate antiapoptotic intracellular signaling pathways.^{27,28} These mechanisms are not necessarily exclusive, but a full clarification of the underlying cause of BMC-induced improvement in car-

diac function is needed to refine their clinical application and maximize their therapeutical potential. Therefore, we sought to elucidate whether BMCs protect the human myocardium from ischemic injury by reducing cell death and to investigate whether the benefit is mediated by activation of the kinases protein kinase C (PKC) and p38 mitogen-activated protein kinase (MAPK), by using a model of simulated ischemia previously characterized in our laboratory.²⁹

Methods**Study Patients and Experimental Preparation**

The study was approved by the local ethics committee, and written consent was obtained from each patient. The right atrial appendage was obtained from patients undergoing elective heart surgery. The experimental preparation used has been previously validated in our laboratory.²⁹ Briefly, upon harvesting, samples were immediately immersed in cold (4°C) Krebs/Henseleit *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid–buffered medium (118 mmol/L NaCl, 4.8 mmol/L KCl, 27.2 mmol/L NaHCO₃, 1 mmol/L KH₂PO₄, 1.2 mmol/L MgCl₂, 1.25 mmol/L CaCl₂, 10 mmol/L glucose, and 20 mmol/L *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid) pre-bubbled with 95% oxygen/5% carbon dioxide (pH 7.4). Muscles were immediately sectioned manually with a skin-graft blade (Swann-Morton Ltd, Sheffield, UK) to slices 300 to 500 μ m thick and weighing 30 to 50 mg. After equilibration under normothermic aerobic conditions (95% oxygen/5% carbon dioxide) for 45 minutes, muscles were subjected to 90 minutes of simulated ischemia (37°C), obtained by bubbling the media with 95% nitrogen/5% carbon dioxide in the absence of glucose and at pH 6.8, followed by 120 minutes of reoxygenation.

Aspiration of Bone Marrow and Preparation of Marrow Cells

With patients under anesthesia and immediately before the initiation of the operation, 40 mL of bone marrow was aspirated from the iliac crest of the same patients donating the atrial appendage by using a bone marrow–harvesting needle (Medical Device Technologies Inc, Gainesville, Fla). The mononuclear fraction was obtained by density gradient. Cells were washed twice and suspended in Krebs/Henseleit medium. The cells were then manually counted by using the Neubauer chamber.

Study Groups

After equilibration, the muscle slices were randomly allocated to the following groups (*n* = 6 per group): (1) simulated ischemia followed by reoxygenation (SI/R), (2) SI/R co-incubated with BMCs (10⁵ cells per milligram of tissue), (3) SI/R with the PKC inhibitor chelerythrine (10 μ mol/L), and (4) SI/R with BMCs and the PKC inhibitor chelerythrine (10 μ mol/L). Time-matched aerobic controls were used for each experiment. Similar experiments were performed by substituting chelerythrine by the p38 MAPK inhibitor SB203580 (10 μ mol/L). Both chelerythrine and SB203580 were obtained from Calbiochem Ltd (Lutterworth, UK).

Commercially available human umbilical vein endothelial cells and keratinocytes were purchased from Cambrex Bioscience (Berkshire, UK). The cells were grown in culture and used after a

few passages. On the day of the experiment, cells were passaged and counted. A total of 10^5 cells per milligram of tissue were incubated with endothelial cells and keratinocytes in addition to BMCs in separate groups ($n = 4$ per group).

Assessment of Tissue Injury

Tissue injury was assessed by measurement of creatine kinase release into the media during the 120-minute reoxygenation period. The enzyme activity was measured by a linked-enzyme kinetic assay by using a commercial assay kit (30-3060/R2; Abbott Laboratories, Diagnostic Division, Kent, UK) and a plate reader (Benchmark; Bio-Rad Laboratories, Hercules, Calif). Results were expressed as international units per milligram of wet weight after subtraction of the aerobic control values.

Assessment of Cell Death

At the end of the experimental protocol, tissues were incubated for 2 minutes on ice with $5 \mu\text{g/mL}$ propidium iodide in 0.1 mol/L trisodium citrate and 20 mmol/L phosphate-buffered saline at pH 7.4 to identify the necrotic nuclei. After this, the muscles were embedded with optical cutting temperature embedding matrix, Tissue-Tek (Agar Scientific Ltd, Essex, UK). Frozen sections were then cut at $8\text{-}\mu\text{m}$ thickness in a Bright cryotome (model OTF, Bright Instrument Co Ltd, Huntingdon, Cambridgeshire, UK) at approximately -25°C , and sections were collected on VECTABOND (Vector Laboratories Ltd, Peterborough, UK)-treated slides. To assess apoptosis, the sections were fixed with 4% paraformaldehyde, washed with 20 mmol/L phosphate-buffered saline at pH 7.4 for 2 minutes, permeabilized in 0.02 mg/mL proteinase K for 10 minutes at 37°C in a humidity chamber, and presensitized for 1 minute in a microwave oven at 800 W in 0.1% Triton X-100 and 0.1 mol/L trisodium citrate at pH 6.0. Terminal deoxynucleotidyl transferase was then used to incorporate fluorescein isothiocyanate-labeled deoxyuridine triphosphate oligonucleotides to DNA strand breaks at the 3'-OH termini in a template-dependent manner (deoxyuride 5'-triphosphate biotin nick end labeling technique) by using a commercially available kit (Roche Diagnostics GmbH, Penzberg, Germany). Finally, to count the total number of nuclei, sections were mounted by using VECTASHIELD mounting medium (Vector Laboratories) and stained with 4',6-diamidino-2-phenylindole.

To assess necrosis, propidium iodide-labeled nuclei were excited with helium-neon laser light at 543 nm , and fluorescence was detected by using an emission range of 680 to 730 nm . For apoptosis, the fluorescein isothiocyanate fluorescence emission range used was 600 to 630 nm ; it was then measured with argon ion fluorescence excitation at 488 nm and detected by laser confocal epifluorescence microscopy.

Analysis was performed with NIH Image software (Scion Corp, Frederick, Md). Fluorescent signals with areas greater than $16 \mu\text{m}^2$ were counted to avoid the inclusion of artifact.

Statistical Analysis

Data were expressed as mean $\pm$ SEM and subjected to analysis of variance followed by post hoc *t* test comparison (Microsoft Excel analysis tool pack; Microsoft Corp, Redmond, Wash).

Results

As shown in Figure 1, A to C, substantial ischemic injury was detected by creatine kinase release and by the degree of cell necrosis and apoptosis. The figure also shows that creatine kinase release was significantly reduced and that cell necrosis and apoptosis were abolished in the groups treated with BMCs, an effect that was reversed by the PKC blocker chelerythrine. Figure 1, D and E, shows representative photomicrographs of necrosis and apoptosis for all the study groups.

Figure 2, A to C, confirms the potent anti-ischemic effect of BMCs seen in the previous study and demonstrates that this benefit is also reversed by blockade of p38 MAPK with SB203580. Figure 2, D and E, also shows representative photomicrographs of necrosis and apoptosis for all the study groups. The observed cardioprotective effect was specific for BMCs because, as shown in Figure 3, the benefit could not be obtained with endothelial cells and keratinocytes.

Discussion

We have demonstrated that BMCs possess potent cardioprotective properties against ischemic injury, with almost complete abolition of cell death by necrosis and apoptosis, a benefit that can be reversed by blockade of the kinases PKC and p38 MAPK. These results suggest that the beneficial effect of BMCs when injected after an acute myocardial infarction is due, at least in part, to increased survival of cardiac tissue. These findings are of importance for cell therapy with BMCs, and their clinical relevance is discussed below.

There is experimental evidence that injection of BMCs into the infarcted myocardium improves cardiac function and reduces infarct size.^{10,11} More recently, there is indication that BMCs also ameliorate cardiac contractility in humans.^{5-9,12} However, it is not clear whether the mechanism of these beneficial effects from the BMCs involves proliferation and differentiation of the injected cells, stimulation of proliferation of putative resident stem cells, or a reduction of myocardial cell death. Animal^{11,30} and clinical^{6-9,12} studies have suggested that BMCs can differentiate into cardiomyocytes, although the capacity of hematopoietic lineage cells to differentiate into heart muscle has been disputed.^{13,14} Although this mechanism could still play a role in tissue repair, the capacity of BMCs to differentiate into cardiomyocytes may occur at a low frequency,³¹ and it might not be sufficient to fully explain the time course and the degree of the benefit observed. Furthermore, few BMCs remain in the heart after injection,³² which also questions the importance of proliferation and differentiation of BMCs into cardiomyocytes. Stimulation of putative resident stem cells by BMCs may also play a role in the regeneration of cardiac tissue, but, again, cells would require some time to

Figure 1. Creatine kinase (CK) release (A) and cell death by necrosis (B) and apoptosis (C) of human right atrium myocardium ($n = 6$ specimens per group) subjected to 90 minutes of simulated ischemia and 120 minutes of reoxygenation in the presence and absence of autologous BMCs and the effect of PKC blockade with chelerythrine (10 pmol/L). * $P < .05$ versus the bone marrow (BM) group. Representative photomicrographs showing necrosis (D) by staining with propidium iodide dye (red) and apoptosis (e) by the deoxyuride-5'-triphosphate biotin nick end labeling technique (green). Necrotic nuclei were determined as a percentage of 4',6-diamidino-2-phenylindole-stained nuclei and subtraction of the percentage of necrotic nuclei from control aerobic sections.

proliferate and fully differentiate into cardiomyocytes. Here we have demonstrated for the first time that in humans, autologous BMCs almost completely abolish myocardial apoptosis and necrosis induced by ischemia. Therefore, the rescue of cardiac tissue from dying may be a plausible explanation for the rapid improvement in function after the administration of BMCs. Our results seem to be specific for BMCs, because other cell types failed to afford cardioprotection. The findings in human myocardium are supported by animal studies in which injection of fractionated BMCs

also reduced apoptosis and decreased the production of proapoptotic proteins.²³⁻²⁵ However, in these animal investigations, it was not possible to separate whether the anti-apoptotic effect was caused by a direct action of the BMCs or was the result of an improvement in blood supply to the ischemic areas by angiogenesis.

A second important finding of our study is that the cardioprotection of BMCs can be reversed by blockade of PKC and p38 MAPK, thus suggesting that these kinases are essential factors in mediating the beneficial effect of these

Figure 2. Creatine kinase (*CK*) release (A) and cell death by necrosis (B) and apoptosis (C) of human right atrium myocardium ($n = 6$ specimens per group) subjected to 90 minutes of simulated ischemia and 120 minutes of reoxygenation in the presence and absence of autologous BMCs and the effect of p38 MAPK blockade with SB203580 (10 pmol/L). * $P < .05$ versus the bone marrow (*BM*) group. Representative photomicrographs showing necrosis (D) by staining with propidium iodide dye (red) and apoptosis (E) by the deoxyuride-5'-triphosphate biotin nick end labeling technique (green). Necrotic nuclei were determined as a percentage of 4',6-diamidino-2-phenylindole-stained nuclei and subtraction of the percentage of necrotic nuclei from control aerobic sections.

cells. In a rat model, Gneccchi and colleagues²⁵ have also suggested that Akt-modified mesenchymal stem cells improve protection of the ischemic heart. It is worth noting that this intracellular signaling pathway of protection by BMCs is also shared by ischemic preconditioning in humans,^{33,34} another potent cardioprotective intervention that per se may induce the recruitment of bone marrow-derived endothelial progenitor cells to the ischemic myocardium.³⁵

Conclusions

The results of this study may have important clinical implications for the treatment of ischemic heart disease and the progression to heart failure. They suggest that the administration of BMCs may prevent myocardial injury and the death of tissue in the acute phase of a myocardial infarction, at a time when massive myocardial necrosis and apoptosis occur.³⁶ Previously it has been suggested that a high ex-

Figure 3. Creatine kinase (CK) release from human right atrial myocardium (n = 4 specimens per group) subjected to 90 minutes of simulated ischemia and 120 minutes of reoxygenation in the absence and in the presence of autologous BMCs, human umbilical vein endothelial cells, and human keratinocytes. * $P < .05$ versus the other groups. IU, International units.

pression of inflammatory factors in the early period of a myocardial infarction may reduce the engraftment of BMCs,³⁷ and, because of this, clinical trials have been designed to administer the BMCs 4 to 9 days after the infarction.^{6,7} Therefore, if BMCs act through a dual mechanism, then it seems logical that they should be applied at more than one time point, initially to reduce the loss of tissue and later to initiate the repair/regeneration of the defective heart muscle.

Our demonstration that the cardioprotection by BMCs during acute myocardial ischemia is mediated by PKC and p38 MAPK is also of clinical relevance because the same beneficial effect could be obtained by selective pharmacologic activation of this signaling pathway without the need for BMC administration. This is of special importance because during the first few hours of a myocardial infarction, the aspiration of the marrow and the separation and injection of the cells may be logistically difficult and impractical. Although these findings need to be confirmed in a clinical setting, it is clear that this approach may reduce the myocardial injury induced by ischemia in acute coronary syndromes and during cardiac surgery. However, it is worth noting that we do not possess the means for the selective manipulation of the kinase-mediated signaling cascades and that, at present, the safest clinical application may be the use of autologous BMCs.

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