Unexpected Severe Calcification After Transplantation of Bone Marrow Cells in Acute Myocardial Infarction

Young-Sup Yoon, MD, PhD; Jong-Seon Park, MD, PhD; Tengiz Tkebuchava, MD, PhD; Corinne Luedeman, BS; Douglas W. Losordo, MD

Background—There has been a rapid increase in the number of clinical trials using unselected bone marrow (BM) cells or the mononuclear fraction of BM cells for treating ischemic heart diseases. Thus far, no significant deleterious effects or complications have been reported in any studies using BM-derived cells for treatment of various cardiac diseases.

Methods and Results—Seven-week-old female Fisher-344 rats underwent surgery to induce acute myocardial infarction and were randomized into 3 groups of 16 rats, each receiving intramyocardial injection of either 7 × 10^5 DiI-labeled total BM cells (TBMCs), the same number of DiI-labeled, clonally expanded BM multipotent stem cells, or the same volume of phosphate-buffered saline in the peri-infarct area. Echocardiography 2 weeks after cell transplantation indicated intramyocardial calcification in 4 of 14 surviving rats (28.5%) in the TBMC group. Histological examination with hematoxylin and eosin staining and von Kossa staining confirmed the presence of extensive intramyocardial calcification. Alkaline phosphatase staining revealed strong positivity surrounding the calcified area suggestive of ongoing osteogenic activity. Fluorescent microscopic examination revealed that acellular calcific areas were surrounded by DiI-labeled TBMCs, suggesting the direct involvement of transplanted TBMCs in myocardial calcification. In contrast, in hearts receiving equal volumes of saline or BM multipotent stem cells delivered in the same manner, there was no evidence of calcification.

Conclusions—These results demonstrate that direct transplantation of unselected BM cells into the acutely infarcted myocardium may induce significant intramyocardial calcification. (Circulation. 2004;109:3154-3157.)

Key Words: myocardial infarction ■ calcium ■ cells ■ transplantation

Heart failure associated with ischemic heart disease is a growing, worldwide epidemic.1,2 Traditionally, the myocardium has been considered to have a very limited capacity for self-regeneration. Therefore, the loss of vasculature and cardiac muscle cells that occurs during myocardial infarction leads to progressive heart failure in up to 50% of survivors.2 Because no currently available therapy is directly targeted toward replacement of lost cardiac tissue, the recent identification of adult stem cells has ignited significant interest in the possibility of using these cells for cardiovascular regeneration.

A growing body of evidence suggests that the adult bone marrow (BM) contains stem and/or progenitor cells, which can give rise to endothelial cells.3–6 With the recent demonstration that administration of whole or selected BM cells7,8 or selected BM-derived circulating cells such as endothelial progenitor cells or hematopoietic stem cells9–13 could induce neovascularization and restore cardiac function after myocardial infarction in animal models, the use of BM cells has been suggested as a possible clinical strategy for the treatment of ischemic heart diseases and heart failure.

Largely because of the ease of harvest and apparent lack of requirement for ex vivo manipulation, there has been a rapid increase in the number of clinical trials using unselected BM cells or the mononuclear fraction of BM cells for treating ischemic heart diseases. Thus far, no significant deleterious effects or complications have been reported in any preclinical or clinical trials using BM-derived cells for treatment of various cardiac diseases.14–18

Our laboratory has been engaged in studies of progenitor and stem cells for treatment of myocardial ischemia and heart failure. Although we have not focused on the use of BM cells per se, we have used these cells as a control in studies focused on the use of other cells; recently, we encountered an unexpected and potentially serious complication. Specifically, these studies revealed that intramyocardial calcification is a potential consequence of injection of BM cells into zones of acute myocardial ischemia.

Methods

Induction of Myocardial Infarction and Cell Transplantation

All procedures were performed in accordance with the guidelines of the Caritas St Elizabeth’s Institutional Animal Care and Use Committee. The study involved 7-week-old female Fisher-344 rats.
Preparation of Total Bone Marrow Cells
The BM cells were harvested from male Fisher-344 rats by flushing tibia and femurs with Dulbecco’s PBS (DPBS) without calcium (Cambrex). The BM was centrifuged at 1300 rpm for 10 minutes to obtain cell pellets. Cell pellets were resuspended in 25 mL of DPBS containing 0.5 mol/L EDTA (DPBS-E). After centrifugation at 1300 rpm for 7 minutes, cells were resuspended in 5 mL of DPBS-E and 20 mL of NH4Cl for induction of hemolysis. After centrifugation and washing with DPBS-E, cells were filtered through a 40-μm nylon filter. Filtered BM was assessed morphologically and tested for viability by Trypan blue staining and for absence of clots, bone spicules, and glass fragments. All cells were labeled with red fluorescent dye, CM-DiI (Molecular Probes), as described previously,10,19 and resuspended in DPBS for injection.

Preparation of Bone Marrow–Derived Multipotent Stem Cells
The procedure of harvest and preparation of BM cells was identical to that described above up to the point of nylon filtering. The filtered total BM cells were then plated in 6-well plates that had been coated with fibronectin (100 μg/mL). The cells were grown in complete DMEM containing 20% of fetal bovine serum (BioWhittaker), 100 U/mL penicillin, and 100 μg/mL streptomycin and 5 mmol/L glucose at 37°C and 5% CO2 for 4 to 6 days; the medium was replaced with fresh complete medium, and the adherent cells were grown to 70% to 80% confluence. Next, the cells were reseeded in 25 mL of DPBS containing 0.5 mol/L EDTA (DPBS-E). After centrifugation at 1300 rpm for 7 minutes, cells were resuspended in 5 mL of DPBS-E and 20 mL of NH4Cl for induction of hemolysis. After centrifugation and washing with DPBS-E, cells were filtered through a 40-μm nylon filter. Filtered BM was assessed morphologically and tested for viability by Trypan blue staining and for absence of clots, bone spicules, and glass fragments. All cells were labeled with red fluorescent dye, CM-DiI (Molecular Probes), as described previously,10,19 and resuspended in DPBS for injection.

Histology
After the rats were euthanized, the aortas were perfused with saline. The hearts were sliced into 4 transverse sections from apex to base, fixed with 4% paraformaldehyde, methanol or frozen in OCT compound and sectioned into 5-μm thicknesses. von Kossa stain was performed to detect abnormal deposits of calcium within the myocardium. PFA-fixed tissue sections were treated with silver nitrate solution; the calcium is reduced by a strong light and replaced with silver deposits, visualized as metallic silver. Procedural details are described elsewhere.21 By von Kossa staining, calcium mineral appears black. Alkaline phosphatase (ALP) staining was performed on frozen tissues after citrate-acetone fixation according to the manufacturer’s instructions (Sigma).21 Frozen sections were examined to detect DiI-labeled transplanted cells within the myocardium after DAPI staining.

Results
During the follow-up, 2 rats died within 1 week in both the TBMC and PBS groups.

Echocardiography
Echocardiography 2 weeks after cell transplantation revealed a bright echogenic mass with acoustic shadowing in the myocardium, suggestive of calcification, in 4 of 14 surviving rats (28.5%) in the TBMC group (Figure, a). No echogenic areas were observed in rats receiving BMSCs or PBS.

Histological Analysis
Rats showing evidence for an echogenic mass were euthanized immediately. Postmortem examination revealed spotty to diffuse white, calcific lesions in peri-infarct and infarct areas (Figure, b). Histological examination with hematoxylin and eosin staining demonstrated deep blue-purple aggregates suggestive of calcium particles interspersed within regions of fibrosis (Figure, c). To further characterize these lesions, von Kossa staining was performed,21 revealing black metallic deposits, typical of calcification (Figure, d). To identify any bone-forming activity around the calcific lesions, ALP staining was performed and revealed strong positivity surrounding the calcified area that suggested the possibility of osteogenic activity, even though definite features of bony tissue were not observed (Figure, e). DAPI staining on the frozen sections, which preserved the red fluorescence of the transplanted DiI-labeled TBMCs, revealed that DAPI-negative acellular calcific areas were surrounded by DiI-labeled BM cells (Figure, f). The observation that the DiI-labeled cells were localized to the calcific rim, where high ALP activity was detected, suggested the involvement of transplanted BM cells in the ongoing calcification. In contrast, in hearts receiving equal volumes of saline or BMSCs delivered in the same manner, there was no evidence of calcification by the same histological techniques.

Discussion
These results demonstrate that direct transplantation of unselected BM cells into the acutely infarcted myocardium may cause significant intramyocardial calcification. These results underscore the potential importance of regulating cellular differentiation of adult stem cells in therapeutic applications. Indeed, there have been concerns raised about the potential for adverse effects, such as tumorigenicity or unregulated differentiation, after stem/progenitor cell transplantation in vivo. It recently has been documented that teratoma formation can occur after embryonic stem cell transplantation.22 More pertinent to the present study, cases of fatal ventricular...
Intramyocardial calcification after transplantation of BM cells into infarcted heart. a, Two-dimensional echocardiography illustrates echogenic density (arrows) with acoustic shadowing suggestive of intramyocardial calcification in anterolateral to inferior wall of the mid-left ventricle (LV) in modified short-axis view (arrows). b, Serial cross sections of LV from base to apex demonstrate white calcific deposits (arrows) throughout LV myocardium. Apical section is heavily calcified. c, Hematoxylin and eosin (H&E) staining shows aggregation of blue-purple densities (arrows) mixed with fibrosis suggestive of calcification. Magnification: left, x40; right, x800. d, von Kossa staining of consecutive myocardial sections counterstained with nuclear fast red reveals deposition of brown-black material (arrows) within the myocardium, indicative of calcification. Magnification: left, x40; right, x800. e, Myocardial section demonstrates strong ALP activity (blue color, arrows) around the calcified area suggestive of possible osteogenic differentiation. Magnification: left, x200; right, x400. f, Fluorescent microscopic images of calcified area demonstrate engraftment of Dil-labeled TBMCs within the myocardium (left panel). DAPI staining shows cellular nuclei as blue (middle panel). Foci of calcification are indicated by DAPI-negative acellular areas (arrows). Merged image (right panel) reveals that calcified areas are surrounded by transplanted Di-labeled TBMCs, suggesting that transplanted BM cells contributed to myocardial calcification.

arrhythmia were reported after intramyocardial skeletal myoblast transplantation.23 Our results provide another cautionary example of the potential toxicity of therapeutic applications of cell transplantation.

The intramyocardial calcification that we observed in our study is notable in that in rat myocardial infarction models, there have been no previous reports about calcification. Moreover, from our own experience with this model in more than 300 cases, we did not observe a single instance of intramyocardial calcification by echocardiography or histological examination.10,19,20 The grossly visible, aggregated calcification could be clinically significant, as it could lead to contractile dysfunction or arrhythmias. In contrast to the calcification occasionally observed after myocardial infarction in humans, which develops in areas of severe transmural infarct or aneurysm,24 the calcification in the BM cell–transplanted hearts was identified in the peri-infarct area or normal myocardium, where myocardial structure was relatively well preserved. These unique features of the calcification identified after TBMC transplantation strongly implicate a role for the BM cells in this process.

The mechanisms by which locally transplanted BM cells might induce calcification remain to be clarified. The possibility of an immunologic response can be excluded on the basis of the use of syngeneic, in-bred rats as donors and recipients. In general terms, it seems likely that this unforeseen complication resulted from the interaction of transplanted cells and the local milieu. Bone marrow is a complex tissue composed of hematopoietic precursors, their differentiated progeny, and a connective tissue network referred to as stroma. The stroma is a heterogeneous mixture of cells including mesenchymal stem cells, multipotent adult progenitor cells, adipocytes, reticulocytes, endothelial cells, fibroblastic cells, and osteoblasts.25–27 It has been well established that the stromal/mesenchymal cells can differentiate into bone, cartilage, fat, and a connective tissue.21,25,28,29 Moreover, now that the plasticity of BM-derived stem cells is well established,26 any BM-derived stem cells, as well as osteoblasts, could be considered as candidates for the induction of local calcification. Second, the local milieu in infarcted myocardium favors the deposition of calcium. Accordingly, the unique combination of highly plastic BM cells and a predilection for calcification in infarcted myocardium may have provided the necessary ingredients for calcification. As alternative mechanisms, the possible interaction between the transplanted marrow cells and the resident calcifying vascular cells in arterial wall30 or the systemic effects of the transplanted cells on calcium metabolism cannot be excluded.

In the present study, we used unselected, filtered BM cells; however, it is equally possible that selected BM cell populations may contain multipotent stem cells capable of contributing to myocardial calcification. The outcome after cell transplantation is likely to be influenced by the method of cell isolation and selection, cell dose, timing of cell transplantation, route of delivery, and clinical setting. As long as the molecular mechanisms governing cellular differentiation in vivo remain undefined, there is a possibility that unwanted differentiation may occur after BM cell transplantation. Given the plasticity26,27 of these cells and the current incomplete knowledge on directed differentiation of adult stem cells.
in vivo, potential benefits and disadvantages must be carefully considered in selecting candidate cells for clinical trials. Our results in murine hearts demonstrate a potential toxicity of uncontrolled differentiation of BM cells in acutely infarcted myocardium. Further efforts must be undertaken to identify the regulatory mechanisms that direct the differentiation of transplanted cells to specific purposes, such as neovascularization, in ischemic heart disease.

Acknowledgments

This study was supported in part by National Institutes of Health grants (HL-53354, HL-60911, HL-63414, HL-63695, HL-66957) and an American Heart Association National Scientist Development Grant (Y.S. Yoon). The authors thank Mickey Neely for her assistance in the preparation of the manuscript.

References

Unexpected Severe Calcification After Transplantation of Bone Marrow Cells in Acute Myocardial Infarction
Young-Sup Yoon, Jong-Seon Park, Tengiz Tkebuchava, Corinne Luedeman and Douglas W. Losordo

_Circulation_. 2004;109:3154-3157; originally published online June 14, 2004; doi: 10.1161/01.CIR.0000134696.08436.65

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/109/25/3154

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org/subscriptions/