Transendocardial Autologous Bone Marrow Mononuclear Cell Injection in Ischemic Heart Failure

Postmortem Anatomicopathologic and Immunohistochemical Findings

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Background—Cell-based therapies for treatment of ischemic heart disease are currently under investigation. We previously reported the results of a phase I trial of transendocardial injection of autologous bone marrow mononuclear (ABMM) cells in patients with end-stage ischemic heart disease. The current report focuses on postmortem cardiac findings from one of the treated patients, who died 11 months after cell therapy.

Methods and Results—Anatomicopathologic, morphometric, and immunocytochemical findings from the anterolateral ventricular wall (with cell therapy) were compared with findings from the interventricular septum (normal perfusion and no cell therapy) and from the inferoposterior ventricular wall (extensive scar tissue and no cell therapy). No signs of adverse events were found in the cell-injected areas. Capillary density was significantly higher \( (P<0.001) \) in the anterolateral wall than in the previously infarcted tissue in the posterior wall. The prominent vasculature of the anterolateral wall was associated with hyperplasia of pericytes, mural cells, and adventitia. Some of these cells had acquired cytoskeletal elements and contractile proteins (troponin, sarcomeric \( \alpha \)-actinin, actinin), as well as the morphology of cardiomyocytes, and appeared to have migrated toward adjacent bundles of cardiomyocytes.

Conclusions—Eleven months after treatment, morphological and immunocytochemical analysis of the sites of ABMM cell injection showed no abnormal cell growth or tissue lesions and suggested that an active process of angiogenesis was present in both the fibrotic cicatricial tissue and the adjacent cardiac muscle. Some of the pericytes had acquired the morphology of cardiomyocytes, suggesting long-term sequential regeneration of the cardiac vascular tree and muscle. (Circulation. 2005;112:521-526.)

Key Words: angiogenesis ■ stem cells ■ heart failure ■ revascularization ■ ischemia

The role of cell-based therapy for the treatment of ischemic heart disease is currently under investigation. In view of the myocardium’s limited capacity to regenerate spontaneously after an ischemic injury, the therapeutic use of exogenous progenitor cells has recently gained increasing interest. In vitro demonstration of functional cardiomyocyte differentiation from bone marrow–derived progenitor cells\(^ 1,2 \) has prompted in vivo studies in animal models, and promising results have been obtained in the repair and regeneration of acute and chronic cardiac muscle lesions. Several types of progenitor cells have been used in experimental models, including bone marrow–derived endothelial and blood cell progenitors, as well as bone marrow mesenchymal progenitors.\(^ 3-6 \)

In humans, similar attempts have been made with surgical, intracoronary, or transendocardial introduction of bone marrow–derived cells to improve cardiac lesions.\(^ 7,8 \) Our group recently reported the results of the first phase I human trial of transendocardial injection of autologous bone marrow mononuclear (ABMM) cells in patients with end-stage ischemic heart disease.\(^ 9 \) We observed a significant increase in perfusion, contractility of ischemic myocardial segments, and

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Phenotype and Functional Characterization of 3×10^7 Cells Injected via a Transendocardial Route*

<table>
<thead>
<tr>
<th>Phenotype, % in ABMM cell fraction</th>
<th>CD45^+ CD34^-</th>
<th>CD45^- CD34^+ HLA-DR^-</th>
<th>T cells (CD4^-)</th>
<th>T cells (CD8^-)</th>
<th>B cells (CD19^-)</th>
<th>NK cells (CD56^-)</th>
<th>Monocytes (CD14^-)</th>
<th>Functional assay, cell No./10^6 ABMM cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFU-GM</td>
<td>802</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CFU-F</td>
<td>1</td>
<td></td>
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</tr>
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NK indicates natural killer; CFU-F, colony-forming-unit fibroblasts; and CFU-GM, colony-forming-unit granulocyte-macrophages.

*After mononuclear fraction purification, cell viability was 98.1%.

functional capacity of the cell-injection recipients. This report presents postmortem cardiac findings from one of these patients.

**Case Report**

The patient was a 55-year-old man with ischemic cardiomyopathy and 2 previous myocardial infarctions (in 1985 and 2000). He began to have symptoms of congestive heart failure 2 years before study enrollment. One year before enrollment, the patient had an ischemic stroke with mild residual right hemiparesis and resultant episodes of chronic tonic-clonic seizures. His risk factors for coronary artery disease included diabetes mellitus type II, hypertension, and hypercholesterolemia.

The patient’s functional capacity was evaluated at baseline by means of a ramp treadmill protocol^{10} with a peak maximal oxygen consumption (VO_2max) of 15.9 mL · kg^{-1} · min^{-1} and a workload of 4.51 metabolic equivalent values (METs). A baseline single-photon-emission computed tomography (SPECT) perfusion study showed a partially reversible perfusion defect in the anterolateral wall, a fixed perfusion defect (scar) in the inferior and posterior walls, and normal perfusion in the septal wall.

Cardiac catheterization revealed a left ventricular ejection fraction of 11%, a 70% ostial and an 85% middle stenosis of the left anterior descending (LAD) coronary artery, an 80% proximal lesion of the left circumflex (LCx) coronary artery, and total occlusion of the first obtuse marginal artery and right coronary artery. The distal segments of the LAD and LCx were diffusely diseased. Owing to the severity and extent of the patient’s coronary disease, he was not considered a candidate for surgical or interventional procedures. At enrollment in our study, he was in New York Heart Association (NYHA) functional class III and Canadian Cardiovascular Society (CCS) angina class III. His serum C-reactive protein level, complete blood count, creatine kinase level, and troponin level were normal at baseline.

The patient received a total of 3×10^7 ABMM cells (the Table) that had been harvested 2 hours before the procedure. With the guidance of electromechanical mapping,^{11,12} the cells were injected transendocardially into the anterolateral wall of the left ventricle. No periprocedural complications were observed.

Noninvasive follow-up evaluation was performed 2 and 6 months after cell therapy. Invasive follow-up evaluation, with cardiac catheterization, was performed at 4 months and revealed no change in coronary anatomy. Symptomatic and functional improvements were noted because the patient returned to NYHA and CCS class I. Holter monitoring showed no malignant ventricular arrhythmias, and signal-averaged ECG parameters remained stable. There was no change in the patient’s medications after cell therapy. There was no change in the global ejection fraction or left ventricular volume on echocardiography. The wall-motion index score (on 2-dimensional echocardiography) improved from 1.94 to 1.65 as contractility increased in 5 segments adjacent to the injected area. Myocardial perfusion, as assessed by SPECT, improved in the anterolateral wall. Mechanical data derived from SPECT showed improvements in regional ejection fraction, wall motion, and thickening. In addition, during ramp treadmill testing, the VO_2max increased from 15.8 to 25.2 mL · kg^{-1} · min^{-1}, and the METs increased from 4.51 to 7.21 at 2 months. At 6-month follow-up testing, the VO_2max reached 31.6 mL · kg^{-1} · min^{-1}, and the METs was 9.03.

From 6 to 11 months after the cell injection procedure, the patient’s cardiovascular condition remained stable. At 11 months, however, he had a tonic-clonic seizure at home and was found in cardiopulmonary arrest by family members.

**Methods**

After signed, informed consent was given by the family, an autopsy was performed, including morphological and immunocytochemical analysis of the heart. This report presents the anatomicopathologic findings about the infarcted areas of the anterolateral ventricular wall, which were the areas that had received bone marrow cell injections. The histological findings from this region were compared with findings from within the interventricular septum (which had normal perfusion in the central region and no cell therapy) and findings from the previously infarcted inferoposterior ventricular wall (which had extensive scarring and no cell therapy).

Immunocytochemical analysis of paraffin sections was performed with antibodies against factor VIII–related antigen (A0082, Dako), vimentin (M0725, Dako), smooth muscle α-actin (M0851, Dako), and CD34 and Ki-67 (NCL-L-End and NCL-Ki67MM1 respectively, Novocastra). Antibodies were reacted with Dako’s EnVision System/HRP, with diaminobenzidine as a chromogen. Frozen sections were fixed, permeated with acetone, and incubated with antibodies for troponin T (T6277, Sigma), smooth muscle α-actin, sarcosomic actinin (A7811, Sigma), and desmin (D1033, Sigma). Antibodies were revealed with anti-mouse or anti-rabbit IgG, F(ab), fragment, conjugated to fluorescein isothiocyanate (1814192 and 1238833, respectively, Boehringer-Mannheim), and counterstained with a 0.1% solution of Evans blue dye (Merck).

Capillary density was monitored by using computerized image analysis (Image-Pro Plus, Media Cybernetics) of randomly selected fields in sections stained with hemotoxylin and reacted with antibody for factor VIII–related antigen (n=108) and randomly selected fields in sections reacted with antibodies for smooth muscle α-actin (n=96). Transverse sections of capillaries identified by staining for factor VIII and pericyte-containing capillaries identified by staining for smooth muscle α-actin were quantified separately. Results were expressed as the mean number of capillaries per square millimeter in the case of factor VIII–stained slides or the number of capillaries containing pericytes in smooth-muscle-α-actin–stained slides. Larger vessels identified by a continuous wall of smooth muscle actin–positive mural cells were excluded. Differences between the anterolateral, septal, and posterior walls were assessed with Kruskal-Wallis ANOVA and the
Student-Newman-Keuls method for pairwise multiple comparison. Results were considered significant if $P$ was <0.05.

Evaluation of the capillary density inside the fibrotic areas within the cell-treated anterolateral wall versus the nontreated posterior wall was performed in 40 selected fields inside all the fibrotic scars, excluding the regions containing cardiomyocytes. Microscope fields (at ×100) of factor VIII–stained slides were digitized, and the number of transverse sections of capillaries per square millimeter of fibrotic zones was assessed. Differences between the treated infarcted zones and the nontreated fibrotic wall were assessed by the Mann-Whitney rank-sum test. Results were considered significant if $P$ was <0.05.

### Results

#### Anatomopathologic Findings

The heart weighed 765 g. There was severe arteriosclerosis with subocclusive calcified atheromata in all coronary arteries, calcification of the pulmonary artery, and moderate atheromatosis of the aorta. The heart cavities were dilated, with hypertrophic walls. There was no evidence of any acute injury or of lesions that could be related to cell injections. A generalized, homogeneous endocardial opacification, affecting all the cardiac internal surfaces, was identified on histological examination as diffuse fibroelastic hyperplasia of the endocardium. Minute focal and punctate scars were observed, mainly in the posterior and anterolateral walls.

The apical zone was thinned and fibrotic. The posterior and apical regions had dense, fibrotic, well-circumscribed scars that separated cardiomyocyte bundles. The septal wall exhibited focal scars interspersed with cardiac fibers in the regions adjacent to the anterior and posterior ventricular walls, but it was devoid of fibrosis in the central region.

The anterolateral ventricular wall that received cell injections had elongated, irregular, and parallel reddish areas throughout. In the same wall, in adjacent regions that did not receive injections, the density and morphology of the fibrotic scars were similar to those of the posterior wall, suggesting that no overt differences were present among the different infarcted areas before cell injections.

#### Morphometric Analysis

The capillary density was significantly higher in the areas of the anterolateral ventricular walls that received cell injections than in the previously infarcted posterior wall ($P<0.0001$) (Figure 1A). The median capillary density in the anterolateral wall was apparently similar to that in the septal wall. However, the broad dispersion of the septal wall data, which may have been due to fibrotic areas in regions close or adjacent to the ventricular walls, generated a statistically significant difference between these 2 groups.

The density of capillaries that contained smooth muscle $\alpha$-actin–positive cells within their walls was also assessed (Figure 1B). The number of such vessels was higher in the anterolateral wall than in the septal and posterior walls ($P<0.0001$). Larger vessels identified by a continuous wall of smooth muscle $\alpha$-actin–positive mural cells were not included in these analyses. The capillary density was significantly higher within fibrotic areas of the anterolateral wall than within fibrotic areas of the posterior wall ($P<0.0001$) (Figure 1C).

#### Histological Findings

The anterolateral wall showed irregular, pale regions of fibrotic tissue intercalated with dark regions of cardiac muscle arranged in roughly parallel, interspersed bands, perpendicular to the ventricular wall plane (Figure 2A). No abnormal cell organization, growth, or differentiation or signs of previous focal necrosis, inflammatory reactions, or tissue repair were found in the region that had received cell injections. Inside the fibrotic tissue, trichrome and picrosirius collagen staining disclosed regions with decreased collagen density, in which a rich vascular tree was present. The anterolateral wall also showed larger central vessels that ramified into smaller ones, parallel to the cardiomyocyte bundles (Figure 2B). In the anterolateral wall, the peripheral zone of fibrotic areas merged into the cardiomyocyte layer and lacked well-defined limits, unlike the fibrotic areas observed in the posterior wall (Figure 2C). No fibrotic tissue was seen in the central area of the septal wall (Figure 2D).
Inflammatory cells were rare in the perivascular region: There were occasional isolated small groups of lymphocytes and, very rarely, granulocytes. At the interface between fibrotic tissue and cardiomyocyte bundles, 2 gradients merged: the decreasing blood vessel diameter and the increasing cardiomyocyte size. Very small cardiomyocytes were seen isolated in the fibrotic matrix adjacent to capillaries in the anterolateral wall, together with a progressively increasing number of fibroblastoid cells that were isolated or interspersed in small groups among the cardiomyocyte bundles.

**Immunocytochemistry Findings**

Immunocytochemical labeling of factor VIII–associated antigen identified a thin endothelial layer of blood vessels in the posterior, septal (Figure 3A), and anterolateral (Figure 3B) ventricular walls. In the anterolateral wall, neither factor VIII nor CD34 was found in the fibroblastoid cell population inside the fibrotic matrix. In the posterior ventricular wall and septum, smooth muscle $\alpha$-actin was readily identified in blood vessel wall cells. This protein was present both in pericytes and in the smooth muscle cells of the thin vessel wall layer (Figure 3C) in the anterolateral wall. The vascular tree of the anterolateral wall showed intense labeling in the blood vessel walls, which had a marked hypertrophy of smooth muscle cells (Figure 3D). The same staining pattern was present in isolated cells located in the perivascular position and in the adjacent region among cardiomyocytes and fibrotic matrix (Figure 3E). Vimentin was present in the endothelial layer of the anterolateral wall, in the perivascular cells, and in cells adjacent to or in close contact with the cardiomyocytes (Figure 4A). These cells frequently formed an extensive network that permeated the fibrotic matrix and the interstitial space among cardiomyocytes.

Desmin was identified in the same cell population. Desmin labeling was less intense in the vascular wall cells and isolated perivascular cells and was more intense in the cells adjacent to cardiomyocytes. On sections perpendicular to the main cardiomyocyte axis, thin desmin-positive fibrils were observed mainly in the submembrane region; on longitudinal sections, a typical transverse banded pattern of desmin was observed (Figure 4C and 4D). Among cardiomyocytes, some of the small cells had strong, peripheral desmin-stained areas (Figure 4D).

In vascular and perivascular cells in the posterior wall and septum, troponin labeling was negative. In the anterolateral wall, troponin labeling was negative in capillary walls but was positive in the adjacent pericapillary pericytes and in cells migrating into the pericapillary matrix (Figure 4E). In larger vessels, troponin-positive cells were observed in the outer cell layers and adventitia, occasionally forming a continuous troponin-positive cell layer around the vessel (Figure 4F). Isolated cells or small groups of troponin-positive cells were found in the area between the fibrotic tissue and cardiomyocytes and inside the adjacent cardiomyocyte bundles. The intensity of labeling increased in the proximity of cardiomyocytes, where some small fibroblastoid cells disclosed a bright cytoplasm.
homogeneously labeled for troponin (Figure 5A). Occasionally, such cells had an increased volume, with troponin labeling restricted to the periphery; the central area was filled by a troponin-negative cytoskeleton similar to the desmin-stained areas in small cardiomyocytes. In mature cardiomyocytes, troponin-specific antibody labeled the peripheral filamentous and sarcomeric cytoskeleton.

Labeling of sarcomeric actinin was similar to that of troponin. However, both pericapillary pericytes and mural blood vessel cells in the anterolateral region were negative for sarcomeric actinin in blood vessels that were deeply embedded in the fibrotic scar matrix and that remained distant from cardiomyocyte bundles. The same cells located in vessels adjacent to or embedded between the cardiomyocyte bundles were positive for sarcomeric actinin, as were the isolated cells or small groups of fibroblastoid cells (Figures 5B and 5C). Some of these cells had increased in size and, in their central region, disclosed sarcomeric actinin that was already organized in the typical banded pattern of sarcomeres (Figures 5D and 5E). In this central region, isolated cells barely larger than pericytes could be observed; only a few sarcomeres were present, suggesting that those isolated cells had acquired some cardiomyocyte characteristics (Figure 5F).

The Ki67 antibody, which identifies cells actively engaged in replication, reacted only rarely with endothelial cells in the posterior wall. In the anterolateral region, the Ki67 antibody also reacted with pericapillary pericytes and with isolated fibroblastoid cells in the surrounding fibrotic matrix (Figure 3F). The overall cell reactivity with Ki67 antibody was relatively low.

Discussion

Accumulating evidence from both experimental animal studies4–6 and human trials7–9 indicates that ABMM cell therapy improves myocardial perfusion in patients with ischemic heart disease. At the same time, clinical stem cell therapy research is focusing more on safety than on efficacy. The present report describes the postmortem study of one patient who underwent transendocardial injection of ABMM cells. Accordingly, the major findings in this report pertain to the procedure’s safety: No abnormal or disorganized tissue growth, no abnormal vascular growth, and no enhanced inflammatory reactions were observed. In addition, some intriguing histological and immunohistochemical findings were documented: (1) There was a higher capillary density in the cell-treated area than in nontreated areas of the heart. (2) A proliferation of smooth muscle α-actin–positive pericytes and mural cells was noted. (3) The aforementioned cells expressed specific cardiomyocyte proteins.

In the postnatal period, new blood vessels form through either vasculogenesis or angiogenesis, in which proliferation of endothelial cells is followed by remodeling of the extracellular matrix and proliferation of blood wall cells.13–15 Endothelial cells can result from bone marrow–derived progenitors (postnatal vasculogenesis) or from the migration and proliferation of endothelial cells from existing vessels (angiogenesis).16 Mural cells such as pericytes and smooth muscle cells can be derived from bone marrow mesenchymal cells (stroma), myofibroblasts, and/or fibroblasts.17 In the neoangiogenic process, pericytes are derived either from cells of adjacent tissues (mobilized by growth factors produced by endothelial cells) or from proliferation of adventitial and pericapillary pericytes and
their distal gliding on the abluminal side of the growing blood vessel’s basement membranes.\textsuperscript{13} The alternative origination of pericytes from mesenchymal stem cells has been proposed and preliminarily confirmed in experimental models.\textsuperscript{18} Pericytes may be essential to achieve a physiological angiogenic process with resultant durable blood vessels. In the present case, when compared with the noninjected regions, the cell-injected wall had marked hyperplasia of pericytes and mural cells. The observed hypertrophic pericytes displayed 2 characteristics: First, although still located in the vascular wall, they expressed specific myocardial proteins and second, they were found in locations that suggested detachment, having migrated into the adjacent tissue and reached proximal cardiomyocytes that were either isolated or in small cell clumps. Closer to cardiomyocytes, the expression of myocardial proteins was enhanced, yielding brighter immunostaining throughout the whole cytoplasm. The significance of these findings remains to be established. However, within the posterior wall, none of the findings was seen, and small blood vessels could only rarely be found.

Notwithstanding the aforesaid described data, the present report has limitations that severely restrict our ability to make conclusions about the role of ABMM cells in myocardial regeneration. The findings could have occurred by chance. It is impossible to exclude the influence of a natural recovery process as the cause for the difference in vascular density between cell-treated and nontreated areas. Comparisons of capillary density among different sections of wall were based on specimens from a single patient. Moreover, this is an isolated, uncontrolled case involving late events after injection of unlabeled cells; it precluded the use of any imaging technique that could have helped to colocalize and identify the presence of stem cell direct descendants within the vessel wall or myocardium. Therefore, the significant difference in vascular density between cell-treated and nontreated areas cannot be extrapolated to a larger population of similar patients. However, the increased vascular density within the cell-injected anterolateral wall accompanied that wall’s improvement in perfusion as assessed by SPECT, whereas all cell-injected anterolateral wall accompanied that wall’s improvement in perfusion as assessed by SPECT, whereas all

Conclusion

At 11-month follow-up evaluation, stem cell therapy was not associated with any adverse histological findings. Morphological and immunohistochemical analysis of the area that underwent ABMM cell implantation suggested that that area had more capillaries than nontreated areas and that ABMM cell therapy was associated with hyperplasia of pericytes, mural cells, and adventitia. Some of these cells had acquired cytoskeletal elements and contractile proteins (desmin, tropinin, and sarcomeric actinin).

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References

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