Bone Marrow–Derived Cardiomyocytes Are Present in Adult Human Heart

A Study of Gender-Mismatched Bone Marrow Transplantation Patients

Arjun Deb, MD; Shaohua Wang, MD; Kimberly A. Skelding, MD; Dylan Miller, MD; David Simper, MD; Noel M. Caplice, MD, PhD

Background—Recent studies have identified cardiomyocytes of extracardiac origin in transplanted human hearts, but the exact origin of these myocyte progenitors is currently unknown.

Methods and Results—Hearts of female subjects (n=4) who had undergone sex-mismatched bone marrow transplantation (BMT) were recovered at autopsy and analyzed for the presence of Y chromosome–positive cardiomyocytes. Four female gender-matched BMT subjects served as controls. Fluorescence in situ hybridization (FISH) for the Y chromosome was performed on paraffin-embedded sections to identify cells of bone marrow origin with concomitant immunofluorescent labeling for α-sarcomeric actin to identify cardiomyocytes. A total of 160 000 cardiomyocyte nuclei were analyzed approximating 20 000 nuclei per patient. The mean percentage of Y chromosome–positive cardiomyocytes in patients with sex-mismatched BMT was $0.23\pm0.06\%$. Not a single Y chromosome–positive cardiomyocyte was identified in any of the control patients. Immunofluorescent costaining for laminin and chromosomal ploidy analysis with FISH showed no evidence of either pseudonuclei or cell fusion in any of the chimeric cardiac myocytes identified.

Conclusions—These data establish for the first time human bone marrow as a source of extracardiac progenitor cells capable of de novo cardiomyocyte formation. **(*Circulation*. 2003;107:1247-1249.)**

Key Words: chimera ■ stem cells ■ myocytes, cardiac ■ transplantation, bone marrow

The concept of the human heart as an organ incapable of self-renewal has recently been challenged by identification of cardiac myocytes of probable extracardiac origin in hearts of patients undergoing sex-mismatched cardiac transplantation.[1–4] The exact source of these cells is currently unclear, but data from experiments in animals support a bone marrow origin.[5] It is important to note that a marked discrepancy in the level of cardiac chimerism has been observed in the gender-mismatched cardiac transplantation setting.[1–4 6,7] Moreover, controversy has arisen with regard to the methodologies used to define chimeric cardiac myocytes in these human studies. Specifically, concerns have recently been raised about the most appropriate techniques required to differentiate (1) true cardiac myocyte nuclei from pseudonuclei,[6] and (2) diploid nuclei from epigenetic phenomena, such as spontaneous cell fusion.[8]

To address the above issues, we used a specific study design and experimental approach. An ideal method to answer the question of bone marrow origin of chimeric myocytes is to analyze hearts of patients who have undergone gender-mismatched bone marrow transplantation (BMT). The presence of Y chromosome–positive cardiomyocytes in the hearts of female patients would conclusively suggest a bone marrow origin for these cells. By using fluorescence in situ hybridization (FISH) combined with immunohistochemistry, we determined the percentage of male cardiomyocytes in autopsy hearts of female patients who had undergone gender-mismatched BMT. To exclude the possibility of false identification of pseudo or fusion nuclei as chimeric cardiomyocytes, additional analyses were performed with the use of basement membrane laminin costaining and chromosome 18 multiploidy analysis with FISH, respectively. Gender-matched BMT patients served as controls.

Methods

Patients and Autopsy Tissue

A review of the Mayo Clinic BMT database and autopsy records identified 4 female patients who had received male donor bone marrow. Female patients who had gender-matched BMT were examined as controls. The Mayo Clinic institutional review board granted approval for the study of human tissue samples.

Combined Immunohistochemical and FISH Analysis

Immunohistochemical analysis of cardiac tissue sections was performed by using a monoclonal antibody against α-sarcomeric actin (Sigma clone 5c5) and a rabbit antibody against laminin (Sigma, St Louis, Mo). The secondary detection used was respectively an anti-mouse antibody conjugated to Cy-3 (Molecular Probes; red) and an anti-rabbit antibody conjugated to Alexa Fluor (Molecular Probes; green). In separate experiments, liver and skeletal muscle tissue from the same subjects was stained with antibodies to human hepatocyte and skeletal muscle actin with the use of monoclonal antibodies

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Clinical Data and Cardiomyocyte Chimerism Analysis of Gender-Mismatched BMT Patients

Patient	Age, y	No. Days From Transplantation to Death	Primary Disease	Cause of BMT Death	Ejection Fraction, %	No. of Y Chromosome–Positive Cardiomyocytes (% Chimerism)		Nuclei Counted
1	32	35	CLL	ARDS	67	60	(0.30)	20 106
2	46	510	CML	BOOP	65	35	(0.17)	20 054
3	44	600	CML	Sudden death	66	51	(0.25)	20 109
4	41	480	ALL	GVHD, BOOP	72	36	(0.18)	20 049
Mean±SD	...	...	...	...	...	0.23±0.06%		...

ARDS indicates adult respiratory distress syndrome; BOOP, bronchiolitis obliterans and organizing pneumonia; GVHD, graft vs host disease; CLL, chronic lymphocytic leukemia; CML, chronic myelogenous leukemia; and ALL, acute lymphocytic leukemia.

(both from Dako). Hepatocytes and skeletal myocytes were visualized using a secondary anti-mouse antibody conjugated to Cy-3.

After immunostaining, FISH was immediately performed as previously described.[3] The X and Y chromosome (CEP X, Y; Vysis Inc; B7322, B-6927) DNA probes used were specific for the α satellite region of each chromosome and labeled with Cy-3 and fluorescein isothiocyanate, respectively. For combined analysis, sarcomeric actin and laminin staining and FISH for Y-chromosome were used. In separate experiments a probe to the centromere of human chromosome 18 (CEP 18 Aqua; light blue dot; Vysis) was combined with X (red dot) and Y chromosome (green dot) analysis to evaluate cell ploidy and exclude cell fusion in the chimeric nuclei identified.

In all cases, FISH signals were enumerated using a Zeiss Axioplan microscope equipped with a triple-pass filter (Vysis). Rigorous criteria were used to identify Y chromosome–positive cardiac myocytes as previously described.[2] Counting of the nuclei and Y chromosome was performed by two independent blinded observers.

Results

Patient Characteristics

The clinical profiles of the 4 female patients who underwent sex-mismatched BMT are shown in the Table. Subjects had a range of hematologic diseases requiring BMT ($2.8\pm0.5\times10^8$ infused cells/kg body weight) and received the same pretransplantation conditioning regimen, which consisted of total body irradiation and cyclophosphamide. All patients were maintained on prednisone, and 2 subjects were maintained on additional cyclosporine and azathioprine after transplantation. Autopsy examination showed no macroscopic or microscopic evidence of inflammation in any of the hearts studied (Figure, A).

Immunofluorescence and FISH Analysis

Histological sections of the left ventricle in gender-mismatched subjects revealed a mean percentage of Y chromosome–positive cardiac myocytes of 0.23±0.06% (Table). The Y chromosome was located eccentrically within the nuclei of chimeric cardiomyocytes (Figure, B and C), and chromosomal ploidy analysis excluded cell fusion (Figure, B, inset). Four female control patients who had undergone sex-matched BMT showed no evidence of Y chromosome positivity in any of the 80 000 cardiomyocyte nuclei analyzed. Basement membrane laminin and sarcomeric actin costaining distinguished true chimeric nuclei with surrounding myocyte cytoplasm from pseudonuclei (Figure, C and D). Male bone marrow–derived hepatocytes and skeletal myocytes were also found in the liver and muscle of female gender-mismatched BMT recipients (Figure, E and F), and mean donor cell chimerism in these tissues was 0.4% and 0.2%, respectively (3000 nuclei analyzed). The detection sensitivity of FISH for Y chromosome in this study was 45%, similar to that cited in previous FISH analysis of tissue sections.[2,4]

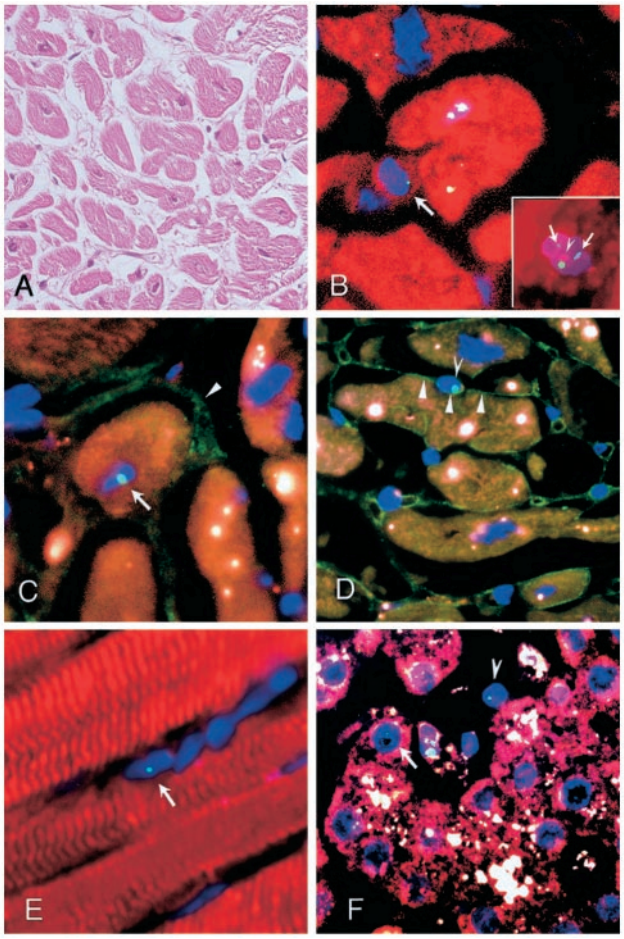
Discussion

These data suggest that adult human bone marrow acts as a source of extracardiac progenitor cells contributing to cardiomyocyte formation. The additional use of laminin costaining and chromosomal ploidy analysis in this study makes the possibility of confusing pseudonuclei or cell fusion events for chimeric myocytes unlikely. The potential origin and phenotype of marrow myocyte precursors in our subjects includes lineage-restricted mesenchymal,[9] hematopoietic,[10] and multipotent adult progenitors[9] and cells of angioblastic lineage.[11]

Physiological stress and tissue injury are known to release cytokines and chemokines, which may promote mobilization of progenitor cells from the bone marrow to the peripheral circulation.[12] Although no patients in our study group had histological evidence of myocardial inflammation, 3 of 4 patients had respiratory complications such as adult respiratory distress syndrome and bronchiolitis obliterans. It is possible that severe tissue injury occurring in these conditions resulted in high levels of circulating cytokines with consequent mobilization of circulating progenitor cells. Interestingly, prior animal experiments showed no detectable engraftment of marrow-derived cells in the absence of myocardial injury.[5] The difference between these animal data and our study may reflect differences in species, duration of study, use of "side population" cells exclusively versus unfractionated bone marrow, or other poorly understood phenomena associated with clinical disease and its treatment.

The consistent levels of chimerism seen at 5 weeks and 20 months after marrow transplantation in our present study suggest a steady-state recruitment of marrow progenitors rather than an initial seeding event early after transplantation. It is noteworthy that a similar recruitment of bone marrow cells occurred in the liver and skeletal muscle as well as the heart, which validates previous animal and human data suggesting multipotent differentiation potential for bone marrow–derived cells.[11,13] It is well known that marrow-derived mesenchymal and hematopoietic stem cells circulate for long periods after transplantation, allowing an equilibrium to be established between circulating and tissue-specific seeding compartments. It is therefore conceivable that low-level recruitment of blood-borne precursors into the myocardium occurs in response to local events in the tissue microenvironment.

Another possibility is that myocardial injury secondary to the pretransplantation conditioning regimen leads by a repair response to recruitment of marrow precursors into the myocardium. This



A, Hematoxylin-and-eosin staining of normal left ventricular myocytes showing no evidence of inflammatory cell infiltrate. B, Cardiomyocyte of female gender-mismatched BMT patient staining positive for α-sarcomeric actin (red) possessing nuclei (blue) positive for Y chromosome (green dot). B, inset, Diploid bone marrow–derived cardiomyocyte nucleus of female gender-mismatched BMT patient showing X chromosome (open arrowhead, red dot), Y chromosome (green dot), and a pair of chromosome 18 (filled arrows, light blue dots) signals; note overlying and surrounding red staining for α-sarcomeric actin. C, Y chromosome–positive true nucleus (blue, green dot; arrow) of bone marrow–derived cardiomyocyte cytoplasm (sarcomeric actin, red) surrounded by basement membrane laminin (green, arrowhead). D, Y chromosome–positive pseudonucleus (open arrowhead) separated from cardiomyocyte (sarcomeric actin, red) by laminin (green-filled arrowheads). E and F, Combined immunofluorescence staining and FISH for Y chromosome in female gender-mismatched BMT subjects showing (E) male skeletal muscle cell (red cytoplasm and blue nucleus with green dot-arrow) and (F) male hepatocyte (red cytoplasm and blue nucleus with green dot-arrow). Note a male cell (open arrowhead) that does not costain with hepatocyte antibody.

scenario seems less likely, however, as the degree of chimerism would be expected to decrease over time and a concurrent "response to injury" would be expected from other blood-borne cells such as leukocytes, neither of which was seen in our study. Furthermore, because all our patients had established hematologic disease before BMT, we cannot automatically infer that chimeric events seen in our study occur under normal healthy conditions, nor can we exclude the possibility that pretransplantation disease may have altered posttransplantation seeding of circulating cells. Finally, we can only speculate on the additional modulating effects of immunosuppressive therapy on bone marrow cell recruitment in our subjects.

The mean percentage of bone marrow–derived cardiac myocytes observed in our subjects was low. It is difficult if not impossible to compare our data with previous chimerism studies both from a clinical and methodological perspective[1–4,7] because it is likely that variables such as chimeric cell detection method, time of study after transplantation, and the presence or absence of inflammation influence the level of myocyte chimerism observed. Finally, while this manuscript was under review, Thiele et al[14] reported 6.4% cardiomyocyte chimerism in a group of male bone marrow transplantation patients, a level more than an order of magnitude greater than our findings. However, the small number of nuclei analyzed and the use of morphology instead of myocyte-specific staining make the identification of chimeric nuclei as true cardiomyocytes less certain in this study.

In conclusion, the present study establishes bone marrow as a contributor to low-level de novo cardiac myocyte formation. The clinical significance of this finding in terms of myocardial regeneration will depend on the success of future efforts to understand and augment the mobilization, homing, and differentiation properties of these cells. Further investigation may also determine whether these cells can be engineered or targeted to diseased myocardium for therapeutic effect.

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