Bone Marrow-Derived Myofibroblasts are Present in Adult Human Heart Valves

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Background and aim of the study: **Endothelial, smooth muscle and cardiomyocyte chimerism has been shown to occur in the human heart. It is currently unknown whether the bone marrow contributes to cellular components of adult human heart valves. Here, it was determined whether bone marrow-derived smooth muscle-like cells (SMLC) are present in the heart valves of adult subjects.**

Methods: **By combining immunofluorescence staining and fluorescence in-situ hybridization (FISH) for X and Y chromosomes, the heart valves of gender-mismatched bone marrow transplant patients were examined for the presence of chimeric cells expressing calponin, a smooth muscle-specific protein. Concomitant staining for CD68 antigen was carried out to exclude cells of a monocytic lineage.**

Results: **The mean percentage of bone marrow-derived SMLC in valves was 0.28 ± 0.03%, with the total proportion of chimeric cells estimated at 0.71 ± 0.05%. The mean proportion of CD68+ cells was 0.33 ± 0.05%. Not a single cell stained doubly for calponin and CD68 antigen.**

Conclusion: **These data establish, for the first time, human bone marrow as a source of progenitor cells contributing to SMLC in adult human heart valves.**

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Bone marrow-derived cells have been shown to contribute to de-novo cardiomyocytes as well as endothelial and smooth muscle cell formation in animal and human subjects. It is currently unclear whether the bone marrow contributes to cellular constituents within other cardiac structures such as heart valves. The aim of the present study was to determine whether any cellular constituents of adult human heart valves are of bone marrow origin.

In order to answer this question, a simple experimental design was adopted. Valves sampled postmortem from patients who had undergone gender-mismatched bone marrow transplantation (BMT) were examined for the presence of bone marrow-derived smooth muscle-like cells (SMLC). Structurally, an adult human heart valve consists of an overlying endothelium with a core extracellular matrix comprising SMLC, also known as myofibroblasts (1). Antibody staining against calponin, along with fluorescence in-situ hybridization (FISH) for X and Y chromosomes, were used to identify bone marrow-derived SMLC. Concomitant immunofluorescence staining for CD68, a monocyte/macrophage marker, was also performed to exclude cells of monocytic lineage. Chimeric, CD68-negative cells, expressing calponin were counted as SMLC of bone marrow origin.

Materials and methods

A review of the Mayo Clinic BMT and autopsy records identified three patients (two males, one female) who had undergone gender-mismatched BMT. One gender-matched female BMT patient served as a control.

Histology

Paraffin-embedded and paraformaldehyde-fixed tissue blocks of mitral and aortic valves (sampled at autopsy) were cut into 5 μm sections, deparaffinized in Citrisolv™, pretreated with 1 mM EDTA, and then subjected to pepsin digestion for antigen retrieval. Immunohistochemical analysis utilizing double antigen staining in the same section was then performed using a monoclonal antibody against the smooth muscle marker calponin (M3556; Dako) and a polyclonal antibody against CD68 (sc-9139; Santa Cruz), as

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described previously (2). The secondary detection used for calponin were goat anti-mouse Alexa fluor 488 (A11001; Molecular Probes) and goat anti-mouse Cy3 (AP124C; Chemicon). A goat anti-rabbit Cy3-tagged antibody (AP136C; Chemicon) was used as the secondary antibody for CD68 detection. After staining, FISH was performed immediately, as described previously (3). The X (CEP X Alpha, 32-112023; Vysis) and Y (CEP Y sat III, 32-112024; Vysis) DNA probes were specific to the (alpha) satellite region of each chromosome, and labeled with fluoroscein isothiocyanate (FITC) respectively. Following hybridization, the sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI) to identify nuclei.

Combined immunostaining and FISH signals were analyzed using a Zeiss Axioplan microscope equipped with a triple-pass filter (Vysis). Calponin-positive, CD68-negative chimeric cells (XX signal in male patients or Y signal in female patients) were identified definitively as SMLC of bone marrow origin.

Results

The clinical profiles of patients who underwent gender-mismatched BMT are detailed in Table I. All patients suffered from a range of hematologic diseases requiring BMT, and received the same pre-transplant conditioning regimen consisting of total body irradiation and cyclophosphamide. One patient who underwent gender-matched BMT served as a control.

Combined immunofluorescence and FISH analysis of the histological sections of heart valves revealed a mean proportion of chimeric SMLC of 0.28 ± 0.03% (Fig. 1). The total proportion of chimeric cells was 0.71 ± 0.05%. The mean percentage of CD68-positive cells was 0.33 ± 0.05%. Not a single chimeric cell stained doubly for CD68 and calponin. About 8,000 nuclei were analyzed using the above analysis, and no false-positive chimerism was identified in the control patient with gender-matched BMT. The detection sensitivity of FISH was approximately 45% in the present study, and correlated closely with values reported elsewhere.

Discussion

The traditional view of the heart as an organ which is incapable of regeneration has undergone considerable change over the past few years. Bone marrow-derived progenitor cells have been shown to contribute to de-novo cardiomyocyte formation in animals as well as human subjects (3-6). A more robust degree of chimerism has been observed with vascular progenitors (7). Circulating endothelial precursors have been shown to contribute to coronary artery endothelium in gender-mismatched heart transplant patients, while bone marrow-derived smooth muscle cells have been identified in human atherosclerotic plaques (7-9). Thus, a growing body of experimental data point to an ability of the bone marrow to regenerate myocytes, endothelial and smooth muscle cells. However, the contribution of the bone marrow to other cardiac tissues such as heart valves is not, as yet, known.

The present results demonstrate, for the first time, the presence of bone marrow-derived SMLC within adult human heart valves. Myofibroblasts comprise the majority of SMLC present within the valve matrix (1), and are likely derived from valvular mesenchymal cells (10). Although the source of these mesenchymal cells is not clear, it is thought that during valve morphogenesis endothelial cells under the influence of various cytokines such as transforming growth factor-β transform into mesenchymal cells. The present data suggest an alternative source of these SMLC. Alternatively, circulating smooth muscle progenitor cells arising from the bone marrow might be a potential source of chimeric SMLC. Consistent with this concept, circulating smooth muscle progenitors recently identified in the present authors' laboratory also express smooth muscle-specific proteins such as calponin, in a pattern similar to myofibroblasts (2).

Table I: Clinical data and smooth muscle-like cell (SMLC) chimerism analysis of gender-mismatched bone marrow transplantation (BMT) patients.

Patient no.	Age (years)	Days from transplant to death	Primary disease	Cause of BMT death	Ejection fraction (%)	Chimeric SMLC (%)	Nuclei counted
1	28	78	MDS	Leukemia relapse	45	0.3	2,100
2	45	121	AML	ARDS	52	0.25	2,000
3	35	37	AML	ARDS	55	0.3	2,100
Mean ±SD					0.28±0.03		

AML: Acute myeloid leukemia; ARDS: Adult respiratory distress syndrome; MDS: Myelodysplastic syndrome.

The mechanisms of mobilization and homing of putative myofibroblast progenitors from bone marrow to heart valves are largely unknown. Conceivably, in the present patients mobilization may have been affected by various cytokines present at high levels secondary to severe systemic illness. Myofibroblasts within heart valves demonstrate significant secretion of metalloproteinases (11), these being key enzymes implicated in promoting the peripheral mobilization of bone marrow stem cells. The homing of such mobilized progenitors may have been influenced by other circulating cytokines such as vascular endothelial

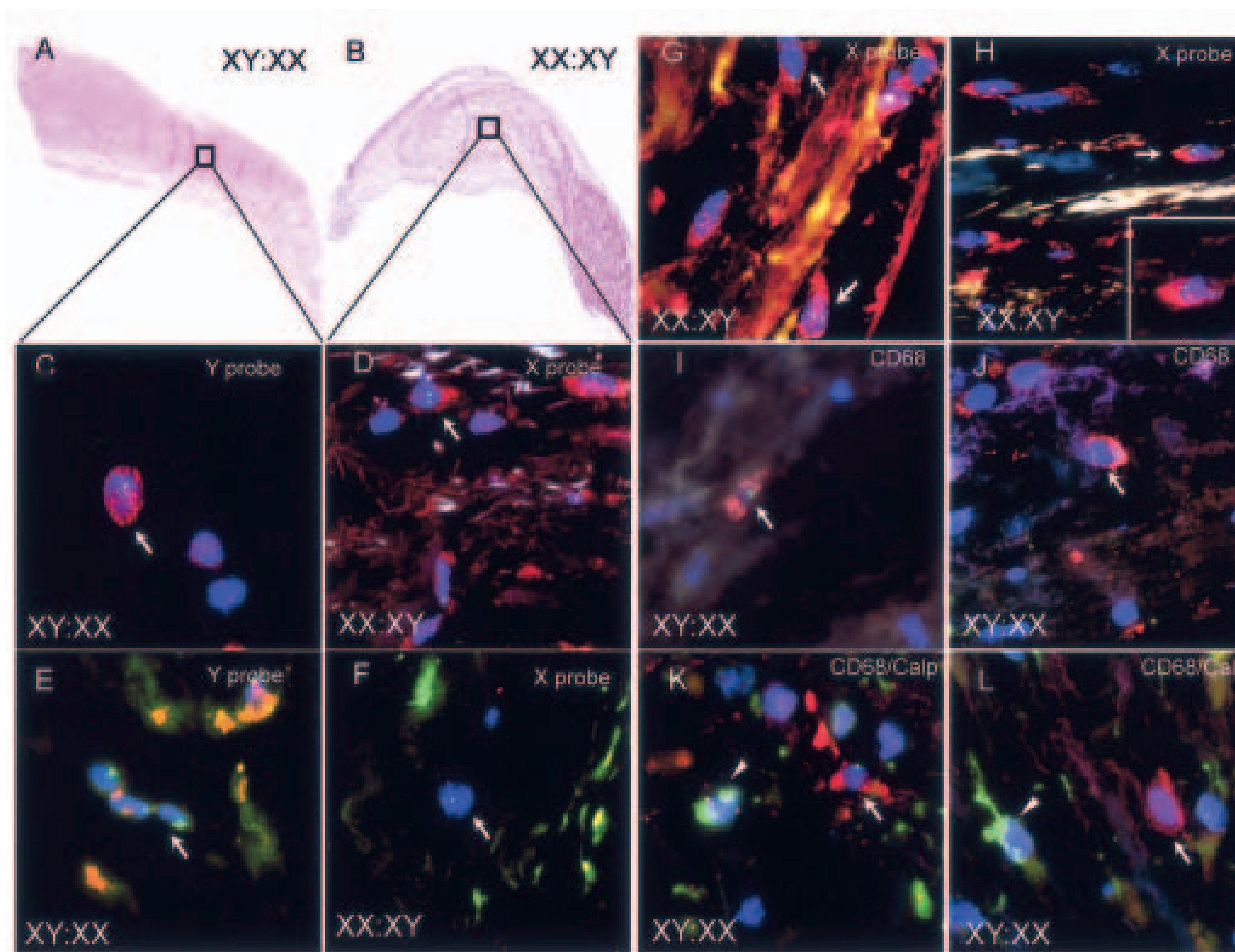

Figure 1: A,B) Hematoxylin and eosin-stained sections of valve tissue from female (A) and male (B) patients who underwent gender-mismatched bone marrow transplantation (BMT). The boxes depict regions of the valves that were subsequently analyzed. C,D) Combined fluorescence in-situ hybridization (FISH), immunofluorescence against calponin, and DAPI counterstaining on representative valve tissue sections. C) Calponin-positive (red), Y chromosome-positive (green dot) smooth muscle-like cells (SMLC) (arrow) in female patient with gender-mismatched BMT. D) Calponin-positive (red), XX chromosome-positive (two green dots), SMLC (arrow) in male patient with gender-mismatched BMT. E,F) Negative control staining for calponin with representative sections stained with isotype IgG antibodies followed by FISH and DAPI staining. Note the absence of staining in chimeric cells (arrow), confirming specificity of calponin staining observed in sections C and D. G,H) Combined FISH, DAPI and calponin immunostaining showing calponin-expressing (red), XX chromosome-positive (green dots) SMLC (arrows) in a male patient with gender-mismatched BMT. The inset in (H) shows a typical chimeric SMLC at high power. I,J) Combined FISH and immunostaining with CD68 shows CD68-positive (red), Y chromosome-positive (green dot) chimeric cells (arrows) in a female patient with gender-mismatched BMT. K,L) Double immunostaining for calponin and CD68 followed by FISH and counterstaining with DAPI in a female patient with gender-mismatched BMT. K) CD68-positive (red), Y chromosome-positive(green dot) cell (arrow) beside a calponin-positive (green), Y chromosome-positive (green dot) SMLC (arrowhead). L) Calponin-positive (green), Y chromosome-positive (green dot) SMLC (arrowhead) beside another CD 68-positive cell (red) which is not chimeric (arrow). Panels (A,B): 10× objective; all other panels: 63× objective.

growth factor which affect key processes such as migration and the differentiation of myofibroblast progenitors during valve formation (10). Alternatively, homing may be determined by local tissue injury. For example, myofibroblasts within the valve express $\alpha 9\beta 1$ integrins which can bind to osteopontin, a ligand which is up-regulated in the valve following tissue injury (12). Up-regulation of these specific molecules following valve injury might potentially regulate recruitment and adhesion of circulating myofibroblast progenitors to the valve matrix.

The presence of bone marrow-derived myofibroblasts within heart valves may have important implications for valve structure and function. These cells synthesize and secrete key extracellular matrix molecules and participate in valve tissue remodeling (1). The number of myofibroblasts within a valve decreases with age, and a reduction in extracellular matrix synthesis along with decreased valve cellularity has been postulated as one potential mechanism underlying degenerative valve disease (13). The concept of bone marrow being a source of myofibroblasts is exciting, as an enhanced mobilization and recruitment of bone marrow-derived myofibroblasts might provide a reservoir for the replenishment of valve myofibroblasts with implications for the maintenance of valve integrity and modulation of valve degeneration.

Furthermore, the present data might have significant implications for a variety of pathological valve conditions, such as calcific valve stenosis. It is currently believed that the pathophysiological processes underlying valve calcification are in many ways similar to those of atherosclerosis and dystrophic calcification of blood vessels. A vascular calcifying cell expressing bone marrow-derived mesenchymal stem cell markers and capable of undergoing multi-lineage differentiation has already been isolated from the artery wall in animal studies (14). A similar calcifying cell has been identified within human heart valves, and is thought to reside within the valve smooth muscle cell population (15). The bone marrow, as suggested by the present findings, might be a potential source of these calcifying myofibroblasts. Pharmacological manipulation of calcifying myofibroblast progenitors may thus serve as a potential future strategy in retarding calcific valve disease.

The biological significance of the low degree of chimerism seen in the present study is not clear. However, myofibroblasts within the valve possess functionally active communicating junctions, and cross-talk between chimeric cells may produce beneficial synergistic biological effects. Alternatively, the degree of chimerism may have been underestimated, as secretory myofibroblasts lacking calponin would have been missed by the techniques employed. Owing to the small number of patients in the present study, the short interval between transplantation and death, immunosuppressive therapy, and the presence of severe systemic illness, it is impossible to speculate on the degree and 'steady-state' contribution of bone marrow to adult heart valves in normal subjects. The clinical translation of the present findings will depend on future efforts to isolate putative myofibroblast progenitors, to elucidate the mechanisms of mobilization and homing of these progenitors, and to determine the roles that they play in the functioning of valves in physiological and disease states. Indeed, the elucidation of these mechanisms may provide us with opportunities for targeted pharmacologic therapy and the modulation of valve function in health and disease.

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