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Original article

Recruitment of bone marrow-derived valve interstitial cells is a normal homeostatic process

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ABSTRACT

Advances in understanding of the maintenance of the cardiac valves during normal cardiac function and response to injury have led to several novel findings, including that there is contribution of extra-cardiac cells to the major cellular population of the valve: the valve interstitial cell (VIC). While suggested to occur in human heart studies, we have been able to experimentally demonstrate, using a mouse model, that cells of bone marrow hematopoietic stem cell origin engraft into the valves and synthesize collagen type I. Based on these initial findings, we sought to further characterize this cell population in terms of its similarity to VICs and begin to elucidate its contribution to valve homeostasis. To accomplish this, chimeric mice whose bone marrow was repopulated with enhanced green fluorescent protein (EGFP) expressing total nucleated bone marrow cells were used to establish a profile of $EGFP^+$ valve cells in terms of their expression of hematopoietic antigens, progenitor markers, fibroblast- and myofibroblast-related molecules, as well as their distribution within the valves. Using this profile, we show that normal (non-irradiated, non-transplanted) mice have BM-derived cell populations that exhibit identical morphology and phenotype to those observed in transplanted mice. Collectively, our findings establish that the engraftment of bone marrow-derived cells occurs as part of normal valve homeostasis. Further, our efforts demonstrate that the use of myeloablative irradiation, which is commonly employed in studies involving bone marrow transplantation, does not elicit changes in the bone marrow-derived VIC phenotype in recipient mice.

1. Introduction

Heart valves are dynamic structures located at the inlet and outlet segments of the left and right ventricles where they maintain unidirectional blood flow during early developmental stages and throughout post-natal life. The post-natal heart valves consist of an overlying endocardium that surrounds an inner extracellular matrix that is organized into three distinct layers: the collagen type I-rich fibrosa, the GAGs-rich spongiosa and the elastic layer, which contains a network of elastic fibers [1–3]. Valve interstitial cells (VICs) are the predominant cell population of the heart valves. They maintain this trilaminar organization of the extracellular matrix of the valve that is required for their proper function. VICs are a heterogenous cell population both in terms of their origin and their phenotype [2,4,5]. While the specific contribution of these cells to valve plasticity during both homeostasis and response to injury is incompletely understood, recent studies have provided new clues to the ability of VICs to respond to changes in composition of their extracellular matrix, cytokine signaling and biomechanical forces [6–8]. Based on these observations, VICs have been subclassified based on their specific contribution to valve development, homeostasis and pathological remodeling [4].

An emerging concept is that cells of extra-cardiac origin contribute to maintenance of the post-natal VIC population. This was first suggested in studies analyzing gender mismatched homograft valve transplants. These studies showed that the donor heart valve fibroblasts were replaced over time by fibroblasts of host origin [9–12]. That circulating cells derived from the bone marrow contribute to the post-natal VIC population was indicated in human studies where donor cells were detected in the valves of individuals that had received gender mismatched bone marrow transplantation [13]. As we have previously shown in a mouse model that transplanted bone marrow-derived $EGFP^+$ cells can give rise to VICs with fibroblast-like properties [14], we sought to further characterize these cells in the adult heart valves under homeostatic conditions. We first examined the distribution of $EGFP^+$ bone marrow-derived cells (BMDCs) within the valves in irradiated-transplanted mice, then profiled these $EGFP^+$ cells for expression of hematopoietic antigens, progenitor markers, fibroblast- and myofibroblast-related molecules. Using this profile generated from irradiated

Abbreviations: EGFP, enhanced green fluorescent protein; VIC, valve interstitial cell; CD, cluster of differentiation; HSC, hematopoietic stem cell; BM, bone marrow; BM-TNC, bone marrow total nucleated cells; LSCM, laser scanning confocal microscope.

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EGFP$^+$ transplanted mice, we show that the valves of control (non-irradiated, non-transplanted) mice have identical BM-derived VIC populations. Collectively, our findings indicate that the recruitment of BM-derived cells that give rise to VICs is a normal homeostatic process.

2. Material and methods

2.1. Animals

All aspects of animal research have been conducted in accordance with guidelines set by the Institutional Animal Care and Use Committee at the Medical University of South Carolina. Ten- to twelve-week old female EGFP-C57BL/6-Ly5.2 mice [15] were used as donors of EGFP$^+$-BM-total nucleated cells (BM-TNCs) for transplantation. Donor cells were transplanted into ten- to fourteen-week old lethally-irradiated male C57BL/6-Ly5.1 mice. Highly engrafted mice were selected for subsequent analyses as previously described. Valves from non-transplanted age-matched Ly5.1 mice were used as controls.

2.2. Reagents

The following primary antibodies were used in this study: rat anti-mouse CD45, rat anti-mouse CD31/PECAM-1, rat anti-mouse CD34, rat anti-mouse CD45R/B220, rat anti-mouse Ly-6G and Ly-6C/Gr-1, hamster anti-mouse CD3e, hamster anti-mouse CD11c, rat anti-mouse I-A/I-E (BD Pharmingen, San Jose, CA), goat anti-mouse CD45 (R&D Systems, Minneapolis, MN), polyclonal chicken anti-GFP (AbCam, Cambridge, MA), rat anti-mouse CD11b/Mac-1, rat anti-mouse F4/80 (AbD Serotec, Raleigh, NC), mouse monoclonal anti-α-smooth muscle actin (Cy3 conjugate, Sigma Aldrich, Minn., MN), and mouse monoclonal anti-Hsp47 (Calbiochem, Darmstadt, Germany). Heat-mediated antigen retrieval in citrate buffer (pH = 6.0) (Vector Laboratories, Burlingame, CA) was used in some immunofluorescence experiments. All fluorochrome-conjugated secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA) and used at a final concentration of 7.0 μg/ml.

2.3. Bone marrow transplantation

A single bolus of 2.0×10^5 bone marrow total nucleated cells (BM-TNC) prepared from EGFP mice was injected into the tail veins of congenic mice that had received a single 850-cGy dose of whole-body irradiation using a 4×10^6 V linear accelerator. The level of blood chimerism was assayed one month after transplantation of EGFP$^+$ cells and flow cytometric lineage analysis of hematopoietic engraftment was performed at two months as previously described [14,17–19]. Four- to six-month old transplanted mice that exhibited high levels of multi-lineage engraftment were used in our study (Supplementary Fig. 1).

2.4. Histological analyses of valve interstitial cells

Hearts from wild-type/non-transplanted (n = 10) and chimeric/transplanted mice (n = 20) were prepared for paraffin sectioning as previously described [14], with the following modifications: fixation was performed by perfusion with zinc-formalin (Anatech LTD, Battle Creek, MI) followed by a 30 minute post-harvest fixation at ambient temperature or overnight at 4 °C. The left lung, left inferior hepatic lobe, spleen, kidney and a portion of the small intestine were isolated from all mice and were used as positive controls for immunofluorescence analyses.

2.5. Whole mount immunolabeling

Harvested valves (Supplementary Fig. 2) were permeabilized in a 0.1% Triton X-100 in PBS for 10 min at ambient temperature. After copious washing in PBS, the tissues were incubated in Background Buster (Innovex Biosciences, Richmond, CA) for 1 h at ambient temperature with gentle agitation. After washing in PBSA (PBS + 0.01% sodium azide) (5 × 20 min), valves were incubated with primary antibodies diluted in 1.0% BSA in PBSA overnight, at ambient temperature with gentle agitation. After washing in PBSA (5 × 20 min), tissues were incubated with coordinate secondary antibodies diluted in 1.0% BSA (in PBSA) overnight at ambient temperature with mixing. After copious washing in PBSA, nuclei were counterstained with Hoechst 33342 (Molecular Probes/Invitrogen, Eugene, OR) diluted in PBSA for 30 min at ambient temperature. Following the final wash procedure, individual valve leaflets were isolated and mounted onto glass slides using Fluoro-Gel (Electron Microscopy Sciences, Hatfield, PA). For sectional analysis, bisected hearts were labeled with primary antibodies, washed in PBSA and fixed in zinc-formalin. Seven-micron sections were cut, cleared in Histoclear II (National Diagnostics, Atlanta, GA), and rehydrated through graded ethanols to PBSA. Primary antibodies were immunolocalized by incubation with coordinate secondary antibodies, diluted in 1.0% BSA in PBS, for 1 h. After copious washing in PBSA, nuclear counterstaining was performed with Hoechst 33342 as described above. After washing, sections were mounted in Fluoro-Gel (as above) and imaged.

2.6. Immunofluorescence analysis

Both whole-mount valve leaflets and sections were imaged using a TCS SP5 AOBS laser scanning confocal microscope (Leica Microsystems, Inc., Exton, PA). For excitation, we used the Diode laser (405 nm, 50 mW continuous wave), the HeNe Orange laser (594 nm, 2.5 mW, continuous wave) and the HeNe Red laser (633 nm, 10 mW, continuous wave). Seven to fifteen optical sections were captured in the Z-axis from every sample with an AOTF 4 channel detector (spectral range: 400–800 nm) and a K-scanner with two independent galvanometers. The thickness of individual optical sections was 2.5 μm for objective 10× (HC PL APO CS, air, numeric aperture: 0.40) and 1.0 μm for 63× (HCX PL APO CS, oil, numeric aperture: 1.4–0.6). Samples were analyzed using bidirectional scanning with four-time lineage average to generate 2048 × 2048 pixel images. Images were exported in tiff format and optical sections were projected using Image J (National Institutes of Health, Bethesda, MD). In the case of high magnification confocal scans (63×), confocal scans three consecutive optical layers were projected. Images were processed using Adobe Photoshop CS4 (Adobe Systems, Inc., San Jose, CA).

3. Results

3.1. Bone marrow-derived GFP$^{\pm}$ exhibit specific distribution cells in the heart valves of transplanted mice

Based on our previous report that EGFP$^+$ HSCs contribute to the valve interstitial cell population in the post-natal mouse valve leaflets [14], we sought to further characterize their phenotype and distribution in the post-natal leaflets using both a model of EGFP$^+$ bone marrow transplantation as well as complementary immunofluorescence analysis in unmanipulated (non-transplanted) mice. First, to evaluate bone marrow-derived cell (BMDC) contribution to the heart valve interstitial cell populations under non-pathological conditions, we transplanted bone marrow total nucleated cells (BM-TNCs) from EGFP$^+$ donor mice into lethally irradiated congenic, non-EGFP adult mice (hereafter referred to as transplanted mice). Two months after BM transplantation, we prepared four-chamber view sections of the hearts from transplanted mice and immunolabeled them with an antibody to GFP.

Fig. 1 provides an overview of the atrioventricular (mitral valve and tricuspid valve, Fig. 1A) and semilunar valves (aortic valve, Fig. 1B) and pulmonary valve, (Fig. 1C) from a transplanted mouse. GFP$^+$ BMDCs are observed throughout the cardiac tissues, as can be seen in the interatrial and interventricular septae (Fig. 1A). Importantly, BMDCs engraft with a specific localization pattern in the heart valves. In the AV valves, the