Journal of Molecular and Cellular Cardiology 49 (2010) 499–507

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Original article

Monocytic fibroblast precursors mediate fibrosis in angiotensin-II-induced cardiac hypertrophy

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ARTICLE INFO

Article history:
Received 19 February 2010
Received in revised form 11 May 2010
Accepted 12 May 2010
Available online 19 May 2010

Keywords:
Fibroblasts
Collagen
Inflammation
Angiotensin
Monocyte chemoattractant protein-1
Monocytes
Progenitor cells

ABSTRACT

Angiotensin-II (Ang-II) is an autacoid generated as part of the pathophysiology of cardiac hypertrophy and failure. In addition to its role in cardiac and smooth muscle contraction and salt retention, it was shown to play a major role in the cardiac interstitial inflammatory response and fibrosis accompanying cardiac failure. In this study, we examined a model of Ang-II infusion to clarify the early cellular mechanisms linking interstitial fibrosis with the onset of the tissue inflammatory response. Continuous infusion of Ang-II resulted in increased deposition of collagen in the heart. Ang-II infusion also resulted in the appearance of distinctive small, spindle-shaped, bone marrow-derived $CD34^+/CD45^+$ fibroblasts that expressed collagen type I and the cardiac fibroblast marker DDR2 while structural fibroblasts were $CD34^-/CD45^-$. Genetic deletion of monocyte chemoattractant protein (MCP)-1 (MCP-1-KO mice) prevented the Ang-II-induced cardiac fibrosis and the appearance of $CD34^+/CD45^+$ fibroblasts. Real-time PCR in Ang-II-treated hearts revealed a striking induction of types I and III collagen, TGF-β1, and TNF mRNA expression; this was obviated in Ang-II-infused MCP-1-KO hearts. In both wild-type and MCP-1-KO mice, Ang-II infusion resulted in cardiac hypertrophy, increased systolic function and hypertension which were not significantly different between the WT and MCP-1-KO mice over the 6-week course of infusion. In conclusion, the development of Ang-II-induced non-adaptive fibrosis in the heart required induction of MCP-1, which modulated the uptake and differentiation of a $CD34^+/CD45^+$ fibroblast precursor population. In contrast to the inflammatory and fibrotic response, the hemodynamic response to Ang-II was not affected by MCP-1 in the first 6 weeks.

1. Introduction

Augmented interstitial or non-adaptive fibrosis in the heart is invariably associated with ventricular remodeling and subsequent cardiac dysfunction and is therefore a common pathological feature of many types of heart failure [1,2]. The development of fibrosis is also often associated with inflammation [3,4]. However, the molecular and cellular mechanisms of non-adaptive fibrosis itself, as well as the contribution of the inflammatory system to the development of fibrosis in the heart are still not well understood.

Traditionally, resident cardiac fibroblasts were thought to be activated by pro-inflammatory processes to proliferate and synthesize collagen that is secreted and deposited in the interstitial space [5,6]. However, recent studies by our laboratory and others describe an important role for the uptake of fibroblast precursor cells of blood-borne, bone marrow-derived origin in pathological interstitial fibrosis [7–10]. We have developed a model in which daily 15-min occlusions of the left anterior descending coronary artery resulted in a fibrotic cardiomyopathy (I/RC) that developed in the absence of myocardial infarction [11]. I/RC depended upon the appearance of a unique population of small, spindle-shaped fibroblasts in the heart that arose from bone marrow-derived (hematopoietic), blood-borne, monocytic fibroblast precursors that expressed CD34 and CD45 [7]. When cultured *in vitro*, these cells were morphologically different than fibroblasts isolated from sham hearts that lacked this cell population [7]. Because there was no myocardial infarction and therefore no myocyte death, I/RC was associated with minimal change in cytokines and chemokines other than a distinctive prolonged rise in monocyte/macrophage chemoattractant protein 1 (MCP-1) expression [11]. MCP-1 is upregulated in inflammatory and fibrotic processes and plays a critical role in the pathogenesis of many fibrotic diseases including cardiac diseases [12]. We extended our observations by subjecting mice with genetic deletion of MCP-1 (MCP-1-KO mice) to I/RC and showed that in these mice the development of the fibrotic cardiomyopathy was obviated [13]. Together with further *in vitro* studies, we demonstrated an obligate role for MCP-1 in the development of non-adaptive fibrosis that resides in its chemoattractive effect on monocytic fibroblast precursors [14]. We

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doi:10.1016/j.yjmcc.2010.05.005

have suggested that this mechanism might constitute a potential link between interstitial fibrosis and inflammation in the heart [7,14].

Since Ang-II generation is associated with almost all occurrences of heart failure and hypertrophy [15–18], and is associated with both inflammation and interstitial fibrosis [19,20], we chose to investigate the involvement of monocytic fibroblast precursor cells in the development of cardiac fibrosis in response to Ang-II. In this study we show that Ang-II infusion induced MCP-1 and the concurrent uptake of monocytes and $CD34^+/CD45^+$ fibroblast precursor cells along with the appearance of marrow-derived $CD45^+$ fibroblasts in the heart. Genetic deletion of MCP-1 obviated the appearance of $CD45^+$ fibroblasts in the heart and markedly reduced cardiac fibrosis. By contrast, deletion of MCP-1 did not alter the appearance of Ang-II-induced hypertension nor cardiac hypertrophy. Our data indicate that the development of Ang-II-mediated fibrosis required induction of MCP-1 and uptake of myeloid fibroblast precursor cells into the heart, but that fibrosis was not the dominant factor in producing the early cardiovascular functional responses to Ang-II.

2. Methods

2.1. Animals

B6.129S4-Ccl2^{tm1Rol}/J (MCP-1-KO) mice (backcrossed to C57BL/6 for >10 generations) and C57BL/6J wild-type (WT) mice (both from Jackson Laboratory) were infused with 1.5 μg/kgBW/min Ang-II via subcutaneously implanted osmotic pumps for 1, 2 or 6 weeks. Control animals were implanted with sterile saline-filled pumps. The investigation conformed with the *Guide for the Care and Use of Laboratory Animals* published by the US NIH. All animals were treated in accordance with the guidelines of the Baylor College of Medicine Animal Care and Research Advisory Committee.

2.2. Cardiac fibrosis

Hearts were embedded in paraffin and sectioned as described earlier [11]. To measure collagen deposition, sections were stained with picrosirius red. Images were scanned and collagen stained areas were calculated as percentages of the total myocardial area. Alpha-smooth muscle actin $(\alpha\text{-SMA})^+$ myofibroblasts and Mac-2^+ macrophage densities were determined by antibody staining [11].

2.3. Immunostaining

After trypsin-induced antigen retrieval and cell permeabilization, heart sections were stained with anti- MCP-1, α-SMA, Mac-2, and CD31 specific antibodies, followed by IgG specific, peroxidase- or fluorescence-conjugated secondary antibodies as described previously [7,11,13].

2.4. Identification of fibroblast populations

Cardiac fibroblasts were isolated and cultured as described previously [7]. Freshly isolated cells were incubated with calcein^{AM}, PE-conjugated anti-CD34, and PE/Cy-5-conjugated anti-CD45. Fluorescence intensities were measured on a Beckman Coulter Epics XL.MCL. Proliferation of cultured cardiac fibroblasts was determined by BrdU incorporation [7]. To normalize data from different experiments, enhanced proliferation in response to serum was expressed as the fold increase compared to cells maintained in serum-free medium. Isolated cardiac fibroblasts were grown on glass slides [7]. Cells were stained with PE-conjugated anti-CD34 or anti-CD45, fixed, permeabilized, then stained with anti-DDR2 or anti-collagen type I, followed by DyLight^{TM} 488-conjugated secondary antibody. Glass slides were mounted with SlowFade Gold containing DAPI. Images were digitally photographed and analyzed.

2.5. mRNA expression

Total RNA was isolated from whole heart with TRIzol reagent and purified via columns (Quiagen RNeasy kit) and cDNA was synthesized. Real-time PCR amplification reactions were performed with iQ SYBR Green Super mix on an iQ5 cycler (BioRad). Gene expression was measured by the ΔΔCT method and was normalized to 18 s ribosomal RNA levels. The data are presented as the fold expression relative to the 1 week control group. Primer sequences: MCP-1: sense 5'-TCCACAAC-CACCTCAAGCACTTC-3' and antisense 5'-GGCATCACAGTCCGAGTCA-CAC-3'; type I collagen: sense 5'-TGTTGGCCCATCTGGTAAAGA-3' and antisense 5'-CAGGGAATCCGATGTTGCC-3'; type III collagen: sense 5'-TGGTCCTCAGGGTGTAAAGG-3' and antisense 5'-GTCCAGCATCACC-TTTTGGT-3'; TNF: sense 5'-CCAGTGTGGGAAGCTGTCTT-3' and antisense 5'-AAGCAAAAGAGGAGGCAACA-3'; TGF-β1: sense 5'-CACTGGAGTTG-TACGGCAGT-3' and antisense 5'-AGAGCAGTGAGCGCTGAATC-3'; 18 s RNA: sense 5'-ACCGCAGCTAGGAATAATGGA-3' and antisense 5'-GCCTCAGTTCCGAAAACCA-3'.

2.6. Cardiovascular parameters

Cardiac function was obtained by 2D-directed M-mode echocardiography (Vevo770; Visual Sonics) and Doppler Ultrasound (Model DSPW, Indus Instruments) as previously described before and after 6 weeks of Ang-II infusion [11]. Functional data were stored and analyzed offline. Blood pressure measurements were obtained by the tail-cuff method (Visitech BP2000) before, and after 1, 2, and 6 weeks of Ang-II infusion.

2.7. Statistical analysis

All data are expressed as mean ± SEM. One-way ANOVA was used to evaluate differences between treatment groups and post-hoc testing (Tukey–Kramer Method) was performed when appropriate (Figs. 1, 3, 5, 6; Table 1). Student's *t*-test was used to evaluate the differences in the % change of cardiovascular parameters (Table 1). A P-value <0.05 was considered statistically significant.

3. Results

3.1. Ang-II infusion resulted in interstitial fibrosis in the heart that is dependent on MCP-1 expression

Compared to saline-treated mice (control), interstitial cardiac fibrosis as determined by histological staining in WT mice was prevalent after 1 week of Ang-II infusion, increased further after 2 weeks, and was still highly prevalent after 6 weeks of Ang-II exposure (Fig. 1A and B). In the Ang-II-treated WT mice, myofibroblast (interstitial $\alpha\text{-SMA}^+$ cells) density peaked after 1 week (Fig. 1C), as did the Mac-2^+ macrophage number (Fig. 1D). When MCP-1-KO mice were infused with Ang-II, the induction of both fibrosis and the accompanying cellular response was obviated. Interstitial collagen deposition in the left ventricle in the Ang-II infused MCP-1-KO mice was comparable to values from untreated control mice (Fig. 1A and B), as was the amount of $\alpha\text{-SMA}^+$ cells (Fig. 1C). The number of Mac-2^+ macrophages in MCP-1-KO hearts was increased by Ang-II infusion compared to control, but was significantly lower than the amount found in Ang-II-treated WT hearts (Fig. 1D). These data indicate that Ang-II infusion induced the deposition of collagen in the heart and that MCP-1 was necessary for this induction.

Immunochemical examination of 2 weeks Ang-II-infused WT hearts indicated that MCP-1 was predominantly expressed around small vessels (Fig. 2). Specifically, MCP-1 was found largely on the surface of CD31 (Fig. 2A) expressing cells and scattered amid the media (Fig. 2A and B) within small vessels, indicating that MCP-1 was present on endothelial and smooth muscle cells. Positive MCP-1 staining was also found on the surface of $CD31^-$ cells within the immediate proximity of