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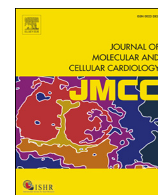

Original article

Endothelin-1 upregulation mediates aging-related cardiac fibrosis

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ABSTRACT

Endothelin-1 (ET-1) plays a major role in regulating myocardial fibrosis in several pathological conditions, such as hypertension and diabetes. Aging is an independent risk factor for myocardial fibrosis. We hypothesized that ET-1 upregulation may be a basis of enhanced collagen synthesis in the senescent fibroblasts resulting in cardiac fibrosis with aging. To examine this hypothesis, we cultured mouse cardiac fibroblasts to passage-30 (P30). β–Galactosidase activity and several other aging markers were markedly increased in P30 (vs. P3) fibroblasts, indicating that these cells were indeed undergoing senescence. Importantly, ET-1 expression was markedly upregulated in P30 (vs. P3) fibroblasts. Of note, estrogen receptor-α (ER-α), an important negative regulator of ET-1, was downregulated in P30 fibroblasts. We also studied aged (130-weeks old, female) mice hearts, and observed that ET-1 was upregulated and ER-α was downregulated in these hearts (vs. 6-week old mice hearts, female). Similar observations were made in the fibroblasts isolated from aged mice hearts. ET-1 upregulation with aging was also seen in ≈70-year old (vs. ≈30-year old) human heart sections. In concert with ET-1 upregulation, the expression of fibronectin and collagens was found to be markedly increased in P30 cardiac fibroblasts in culture, fibroblasts isolated from the aged mice hearts, and in aged human hearts. Interestingly, inhibition of ET-1 in the senescent P30 fibroblasts by 2 different strategies (the use of siRNA and the use of endothelin converting enzyme inhibitors) markedly suppressed expression of fibrosis signals. Further, treatment with synthetic ET-1 enhanced fibronectin and collagen expression in P3 cardiac fibroblasts. These observations in mice and human hearts suggest that aging-related cardiac fibrosis is, at least partially, dependent on the upregulation of ET-1.

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1. Introduction

Heart failure is a major problem affecting a large number of patients around the world [1,2]. Excessive fibrosis, a hallmark of late stage heart failure, occurs in longstanding hypertension and myocardial ischemia. Prevalence of heart failure increases with age, and aging itself is an independent risk factor for cardiac fibrosis [3,4]. Many strides have been made in understanding etiologic basis of fibrosis. The aging-related changes in the aged human heart include myocardial hypertrophy, fibrosis and diastolic dysfunction [5]. Aging-dependent collagen accumulation in heart would be expected to lead to a progressive increase in ventricular stiffness and impaired diastolic function [1].

Endothelin-1 (ET-1) is a potent vasoconstrictor peptide that is mainly produced by vascular endothelial cells in response to hypoxia, oxidized LDL, Ang II, pro-inflammatory cytokines, and bacterial toxins [6]. Activation of estrogen receptor-α (ER-α), a nuclear receptor for estrogen, has been shown to negatively regulate ET-1 expression [7,8]. ET-1 is also produced by other cell types, such as smooth muscle cells, epithelial cells, cardiomyocytes, fibroblasts and certain cancer cells [6,9,10]. Mature ET-1 is formed from pre-pro-ET-1 via a 39-amino acid intermediate, big ET-1 [6,11]; this process requires several enzymes, including the endothelin converting enzymes (ECEs) [6]. Fibroblasts from different tissues can generate significant amounts of ET-1 [12–15], and the exposure of fibroblasts to ET-1 in turn stimulates collagen synthesis [16]. Excessive production of ET-1 has been incriminated in the development and progression of pulmonary and systemic hypertension and other cardiovascular disorders such as atherosclerosis [17,18]. It is of note that ET-1 secretion has been shown to promote fibroblast proliferation and cardiac fibrosis in pathological states, such as diabetes and hypertension [18,19]; however, the role of ET-1 in aging-related heart fibrosis is still not clear. In our preliminary study, we observed ET-1 expression to be hugely increased in senescent cardiac fibroblasts and aged mice hearts. So, we hypothesized that excess ET-1 expression is a major component of the pathogenesis of cardiac fibrosis with aging. This study was designed to address hypothesis.

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To address our hypothesis, we established senescent cardiac fibroblasts by serial passages. We assessed ET-1 expression and fibrosis signals in young (passage-3, P3) and senescent (passage-30, P30) fibroblasts, and thereafter in the aged (130-week old) mice hearts and in the fibroblasts isolated from aged mice hearts. To further confirm the observation of upregulated ET-1 expression in the mice hearts and senescent fibroblasts, we evaluated the expression of ET-1 and collagens in the aged human hearts. Lastly, we studied the role of ET-1 upregulation in fibrogenesis with the use of ET-1 inhibition and over-expression strategies.

2. Materials and methods

2.1. Cardiac fibroblast culture

Ten-week-old C57BL/6 female mice were anesthetized with sodium pentobarbital (80 mg/kg, i.p), and their hearts quickly removed and used for isolation of cardiac fibroblasts. Isolation and culture of mice cardiac fibroblasts were performed as described [2]. The cells were cultured for up to passage-30 (P30). Experiments described subsequently in cultured fibroblasts were mainly performed in passage-3 (P3) and P30 cardiac fibroblasts.

2.2. Collection of heart tissue from mice and humans

Hearts were collected from young (6-week-old) and old (130-week-old) C57L/6 female mice following pentobarbital anesthesia. A part of the hearts (from 5 mice) was frozen at −80 °C for molecular biology studies, and another part was fixed in 10% neutral formaldehyde for histological analysis. The left ventricular tissue was utilized to extract proteins or to perform histological analysis. All experimental procedures were conducted in accordance with protocols approved by the Institutional Animal Care and Usage Committee, and complied with all local, state and federal guidelines.

Heart blocks were obtained from the Department of Pathology archives of the affiliated University of Arkansas for Medical Sciences. The young (≈30 years old, n = 3) and the elderly (≈70 years old, n = 3) subjects from whom the tissues were obtained died of accidents and were free of any obvious cardiac disorder. The protocol for collection of the archived tissue sections was approved on an exempt basis by the Institutional Review Board. The human study conformed to the Helsinki declaration.

2.3. SA-β-Gal activity

β–Galactosidase (SA-β-Gal) activity, as a marker of aging, was measured in cultured cardiac fibroblasts and mice heart tissue by an X-gal Staining Kit (Cell Signaling Technology, Danvers, MA; cat no: #9860), as per manufacturer's instructions. In brief, P3 and P30 cardiac fibroblasts (3×10^5/well) were plated in 12-well plates and cultured overnight. After washing with PBS, the cells were fixed in 1X Fixative Solution (2% formaldehyde and 0.2% glutaraldehyde in PBS) for 15 min at room temperature. After washing with PBS twice, the cells were incubated with SA-β-Gal staining Solution (930 μl 1X Staining Solution, 10 μl Staining Supplement A, 10 μl Staining Supplement B and 50 μl 20 mg/ml X-gal in DMF; pH 6.0) at 37 °C overnight in a dry incubator. The cells were viewed and imaged under a microscope while the SA-β-Gal Staining Solution was still in the wells. The SA-β-Gal positive staining in fibroblasts and in heart tissue was analyzed by Image *J* software.

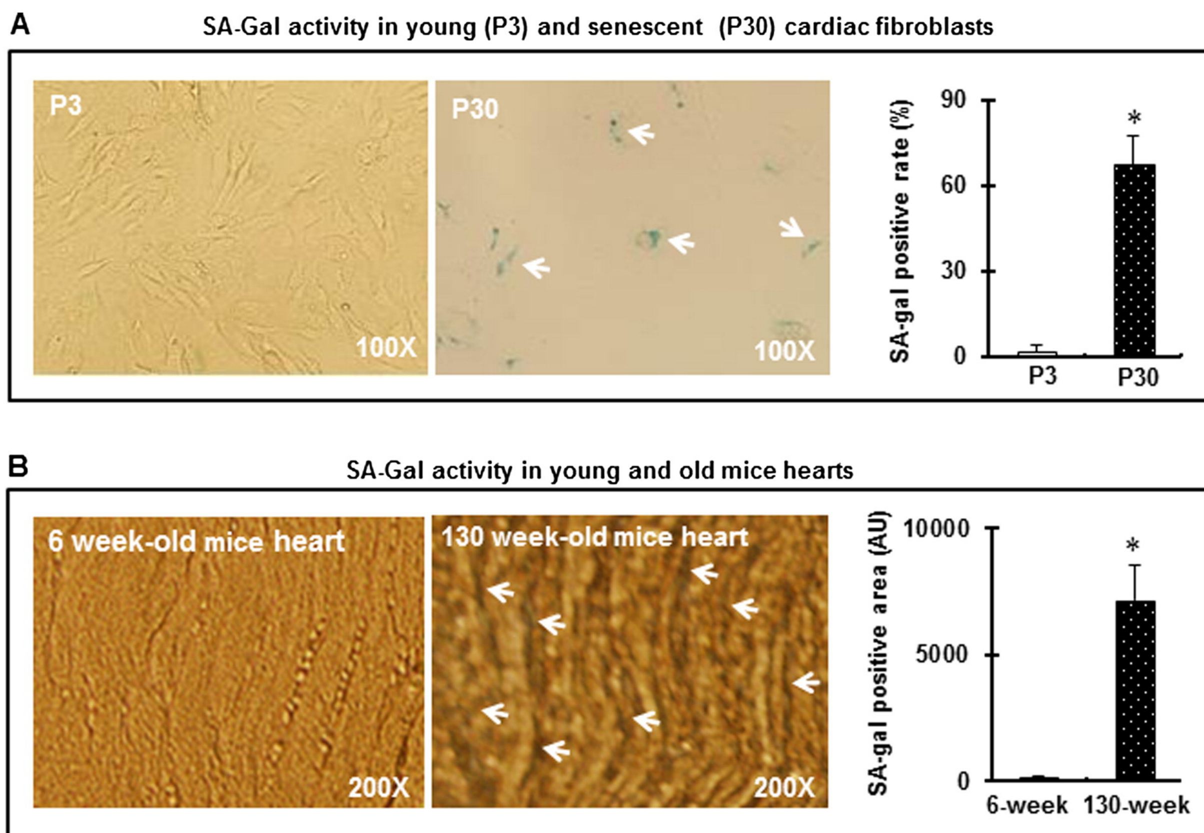

Fig. 1. Representative images of SA-Gal activity in passage-3 (P3) and passage-30 (P30) mouse cardiac fibroblasts (A) as well as in young (6-week old) and old (130-week old) mice hearts (B). Graphs show data as mean (±SD); n = 5 for cultured fibroblasts; n = 5 for mice hearts; and at least 5 fields/sample were calculated. *P < 0.01 vs. P 30 fibroblasts or young mice hearts.

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