JOURNAL OF THE AMERICAN COLLEGE OF CARDIOLOGY
© 2016 BY THE AMERICAN COLLEGE OF CARDIOLOGY FOUNDATION
PUBLISHED BY ELSEVIER

VOL. 67, NO. 17, 2016
ISSN 0735-1097/$36.00
http://dx.doi.org/10.1016/j.jacc.2016.02.047

Essential Role for Premature Senescence of Myofibroblasts in Myocardial Fibrosis

Kathleen Meyer, MSc,[a,b] Bettina Hodwin, MSc,[a] Deepak Ramanujam, PhD,[a,b] Stefan Engelhardt, MD, PhD,[a,b] Antonio Sarikas, MD[a,b]

ABSTRACT

BACKGROUND Fibrosis is a hallmark of many myocardial pathologies and contributes to distorted organ architecture and function. Recent studies have identified premature senescence as a regulatory mechanism of tissue fibrosis, but its relevance in the heart remains to be established.

OBJECTIVES This study investigated the role of premature senescence in myocardial fibrosis.

METHODS Murine models of cardiac diseases and human heart biopsies were analyzed for characteristics of premature senescence and fibrosis. Loss-of-function and gain-of-function models of premature senescence were used to determine its pathophysiological role in myocardial fibrosis.

RESULTS Senescence markers $p21^{CIP1/WAF1}$, senescence-associated ß-galactosidase (SA-ß-gal), and $p16^{INK4a}$ were increased 2-, 8-, and 20-fold ($n = 5$ to 7; $p < 0.01$), respectively, in perivascular fibrotic areas after transverse aortic constriction compared with sham-treated control subjects. Similar results were observed with cardiomyocyte-specific β1-adrenoceptor transgenic mice and human heart biopsies. Senescent cells were positive for platelet-derived growth factor receptor-α, vimentin, and α-smooth muscle actin, specifying myofibroblasts as the predominant cell population undergoing premature senescence in the heart. Inactivation of the premature senescence program by genetic ablation of p53 and $p16^{INK4a}$ (*Trp53*$^{-/-}$ *Cdkn2a*$^{-/-}$ mice) resulted in aggravated fibrosis after transverse aortic constriction, when compared with wild-type control subjects ($49 \pm 4.9\%$ vs. $33 \pm 2.7\%$; $p < 0.01$), and was associated with impaired cardiac function. Conversely, cardiac-specific expression of CCN1 (CYR61), a potent inducer of premature senescence, by adeno-associated virus serotype 9 gene transfer, resulted in ~50% reduction of perivascular fibrosis after transverse aortic constriction, when compared with mock- or dominant-negative CCN1-infected control subjects, and improved cardiac function.

CONCLUSIONS Our data establish premature senescence of myofibroblasts as an essential antifibrotic mechanism and potential therapeutic target in myocardial fibrosis. (J Am Coll Cardiol 2016;67:2018–28) © 2016 by the American College of Cardiology Foundation.

Fibrosis is a hallmark of most myocardial pathologies with limited treatment options (1). Whereas extracellular matrix (ECM) production during wound healing provides structural integrity to damaged tissue due to the negligible self-regenerative capacity of the mammalian heart, excessive fibrosis imposes detrimental effects and may result in scarring and loss of heart function (1). The molecular mechanisms that regulate ECM homeostasis and fibrogenesis are complex and incompletely understood. A signaling network of growth factors and cytokines is thought to cooperate to induce activation of cardiac fibroblasts (CF) and regulate the expression of ECM components and matricellular proteins (e.g., matrix metalloproteinases) to modulate the fibrotic response (2). Moreover, it is

Listen to this manuscript's audio summary by *JACC* Editor-in-Chief Dr. Valentin Fuster.

From the [a]Institute of Pharmacology and Toxicology, Technische Universität München, Munich, Germany; and [b]Deutsches Zentrum für Herz-Kreislauf-Forschung (DZHK), partner site Munich Heart Alliance, Munich, Germany. This study was supported by a research grant from the German Research Foundation (SA 1706/3-1 to Dr. Sarikas), a Marie Curie International Reintegration grant (256584 to Dr. Sarikas), and a Laura-Bassi stipend of Technische Universität München (to K. Meyer). The authors have reported that they have no relationships relevant to the contents of this paper to disclose.

Manuscript received July 17, 2015; revised manuscript received February 8, 2016, accepted February 15, 2016.

increasingly appreciated that the cellular microenvironment and cross-talk between different cell populations contribute to regulation of fibrosis (3).

Recent studies have uncovered a critical role for premature cellular senescence in tissue remodeling (4). In contrast to replicative senescence, originally characterized in human fibroblasts undergoing telomere erosion in culture (5), premature senescence is an irreversible form of cell-cycle arrest that can be triggered by various cellular stresses, including deoxyribonucleic acid damage, oncogene activation, and oxidative stress (6). Seminal studies have established premature senescence as an essential tumor-suppressive mechanism by stalling proliferation of oncogene-harboring cells at risk of neoplastic transformation (7–9). Additionally, senescent cells are found in aged tissues (4,10), although their role in this context has not been fully investigated. Senescent cells remain viable and metabolically active, but they are unable to proliferate despite the presence of mitogens. In addition to cell-cycle arrest, senescent cells exhibit the up-regulation of $p16^{INK4a}$, $p21^{CIP1/WAF1}$, and senescence-associated ß-galactosidase (SA-ß-gal) that distinguishes them from most quiescent cells (11,12). Furthermore, senescent cells are characterized by the up-regulation of secreted proteins and microenvironment modulators that comprise the senescent-associated secretory phenotype or senescence messaging secretome (13,14).

SEE PAGE 2029

More recently, senescent cells were shown to accumulate in liver and skin damage, dampening the fibrotic response through the expression of antifibrotic proteins (15,16). This suggests that senescent cells may arise to limit fibrosis during tissue repair, although this has not been examined in the heart.

In this study, we analyzed different murine models of cardiac diseases and human heart biopsies for characteristics of premature senescence and fibrosis. In addition, loss-of-function and gain-of-function models were used to determine an essential role of premature senescence in restraining cardiac fibrosis.

METHODS

Transverse aortic constriction (TAC) was performed on 8-week-old male C57BL/6N mice as described previously (17). In sham surgery, only the chest was opened, but no ligation of the aorta was performed. Cardiac dimensions and function were analyzed by pulse-wave Doppler echocardiography before TAC/sham surgery and before the animals were euthanized. ß1-adrenoceptor transgenic mice (18) on Friend Virus B NIH (FVB/N) background were analyzed at the age of 2.5, 5, and 10 to 12 months. We also obtained p53 knockout mice (B6.129-*Trp53*^{tm1Brd} N12) and CDKN2 knockout mice (B6.129-*Cdkn2a*^{tm1Rdp}/Nci); the latter carry a targeted deletion of exons 2 and 3 of the INK4a/ARF locus that eliminates both p16 (Ink4a) and p19 (Arf). *Trp53*$^{-/-}$ *Cdkn2a*$^{-/-}$ compound mutant mice were generated by crossbreeding *Trp53*$^{+/-}$ and *Cdkn2a*$^{+/-}$ mouse strains. For cardiotropic expression of exogenous CCN1, 3-week-old male wild-type (WT) mice received adeno-associated virus serotype 9 (AAV9)-CCN1 or AAV9 dominant-negative CCN1 mutant (AAV9-CCN1-DN) (1×10^{12} genome copies per mouse) or iodixanol by tail vein injection before TAC. All animal studies were performed in accordance with the relevant guidelines and regulations of the responsible authorities.

Myocardial fibrosis and premature senescence were determined as described previously (19,20). For further details, see the Online Appendix.

STATISTICAL ANALYSIS. Data are shown as mean ± SEM. Statistical analysis was performed with Prism (version 6, GraphPad Software, Inc., La Jolla, California). Data distribution was assessed by a Shapiro-Wilk test for normality. Differences between 2 means were assessed by a 2-tailed paired or unpaired Student *t* test. Differences among multiple means were assessed by 1-way or 2-way analysis of variance followed by the Bonferroni correction as indicated. A p value of <0.05 was considered significant.

ABBREVIATIONS AND ACRONYMS

AAV9 = adeno-associated virus serotype 9
CF = cardiac fibroblasts
CM = cardiomyocyte
DN = dominant-negative
ECM = extracellular matrix
RB = retinoblastoma
SA-ß-gal = senescence-associated ß-galactosidase
TAC = transverse aortic constriction
TG = transgenic
WT = wild type

RESULTS

ACCUMULATION OF SENESCENT CELLS IN FIBROTIC HEART TISSUE. To investigate the relationship between fibrosis and premature senescence in the heart, we analyzed 2 established animal models of cardiac fibrosis and human heart biopsies for markers of cellular senescence. In TAC, an experimental model for pressure overload-induced cardiac hypertrophy and fibrosis (17), fibrosis typically originates from areas surrounding the coronary arteries and spreads throughout the myocardium. Perivascular fibrosis was significantly increased in TAC hearts when compared with hearts of sham control subjects 2 and 6 weeks after surgery (~1.7- and ~1.3-fold, respectively) (**Figure 1A**, Online Figure 1). To detect senescent cells, heart sections were stained for proliferation marker KI-67 and a panel of senescence markers were used that included SA-ß-gal, $p16^{INK4a}$, and $p21^{CIP1/WAF1}$. SA-ß-gal is the most established