Cell Cycle Perturbation Induces Collagen Production in Fibroblasts

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Summary
Myocardial infarction (MI) occurs when the heart muscle is severely damaged due to a decrease in blood flow from the coronary arteries. During recovery from an MI, cardiac fibroblasts become activated and produce extracellular matrices, contributing to the wound healing process in the damaged heart. Inappropriate activation of the fibroblasts leads to excessive fibrosis in the heart. However, the molecular pathways by which cardiac fibroblasts are activated have not yet been fully elucidated.

Here we show that serum deprivation, which recapitulates the cellular microenvironment of the MI area, strikingly induces collagen production in C3H/10T1/2 cells. Based on transcriptomic and pharmacological studies, we found that cell cycle perturbation is directly linked to collagen production in fibroblasts. Importantly, collagen synthesis is increased independently of the transcriptional levels of type I collagen genes. These results reveal a novel mode of fibroblast activation in the ischemic area, which will allow us to gain insights into the molecular mechanisms underlying cardiac fibrosis and establish a basis for anti-fibrotic therapy.

Key words: Fibrosis, Serum deprivation

Cardiac fibroblasts are activated in the area of myocardial infarction (MI) in the heart, producing collagen or other extracellular matrices (ECM). Fibrotic scars replace the damaged cardiomyocytes and support the structural integrity of the heart, in a manner called "reparative" or replacement fibrosis. At the same time, cardiac fibroblasts can be inappropriately activated in hypertensive or diabetic patients, leading to "reactive" or excessive fibrosis. As inappropriate accumulation of ECM can restrict ventricular function or elicit cardiac arrhythmias, ECM is currently becoming an established therapeutic target in heart diseases. Anti-fibrotic therapy in the treatment of heart diseases must target excessive fibrosis while leaving replacement fibrosis unaffected. Hence, it is crucial to understand the molecular processes by which fibroblasts are activated in the ischemic area. TGF-β is a well-known profibrotic factor which increases the transcript levels of type I collagen, leading to the accumulation of ECM. Moreover, TGF-β activates cardiac fibroblasts and acts as a master cytokine in ECM production. However, knowledge of fibroblast activation signals other than TGF-β remains incomplete.
Figure 1. Serum deprivation accelerates collagen production in C3H/10T1/2 cells. A: The representative image shows the results of Sirius Red staining of the culture media. B: C3H/10T1/2 cells were cultured for 24 hours in the presence or absence of FBS or TGF-β1 (50 ng/mL). Data represent the colorimetric analysis of the collagen content in each culture medium. C: Data show the relative transcript levels of type 1 collagen genes (Col1a1 and Col1a2), Actin, alpha 2, smooth muscle, aorta gene (Acta2) and Serine (or cysteine) peptidase inhibitor, clade E, and member 1 gene (Serpine1). The data represent the mean ± SD. Two-tailed unpaired Welch’s t-test was used for the statistical analysis. *P < 0.05.
Figure 2. Serum deprivation decreases the transcript levels of cell cycle-related genes. A: Transcriptome analysis of C3H10T1/2 cells was performed in the presence or absence of FBS. The flow chart illustrates the strategy for identifying the genes whose expression is affected in a serum-deprived environment. B: Gene Ontology categories of down-regulated genes in a serum-deprived condition (P<0.05). Red color indicates cell cycle-related terms. C: Data show the transcript levels of cell cycle-related genes (Ccnb2, Ccnb1 and Gmnn). The data represent the mean ± SD. Two-tailed unpaired Welch’s t-test was used for the statistical analysis. *P<0.05.

(GIBCO; Life Technologies, Carlsbad, CA, USA) with 10% fetal bovine serum (HyClone Laboratories Inc., South Logan, UT, USA), 2 mmol/L L-glutamine (Sigma-Aldrich, Tokyo), 100 U/mL penicillin and 100 mg/mL streptomycin (GIBCO) at 37°C in a 5% CO2 and 95% humidified atmosphere.

Collagen assay: C3H/10T1/2 cells were cultured onto 6-well plates at 4 × 10^4 per well for 24 hours. Cells were cultured in the presence or absence of 10% FBS, 50 mg/mL recombinant human TGF-β1, or 10mM methotrexate (Sigma-Aldrich) for another 24 hours. The culture media were collected, and collagen content was determined using a Sirus Red Total Collagen Detection Kit (Chondrex # 90626, Redmond, WA, USA) according to the manufacturer’s protocol. Data were normalized by total cell number. G1 synchronization was performed by the double-thymidine block method. Briefly, C3H/10T1/2 cells were seeded onto 6-well plates at 2 × 10^4 per well. Cells were cultured in the presence of 2 mM thymidine (Sigma-Aldrich) for 18 hours and then released to culture for 9 hours. Cells were treated with 2 mM thymidine again for another 18 hours.

RNA isolation and quantitative PCR: Total RNA was isolated from cells using an RNeasy mini kit (Qiagen, Tokyo) according to the manufacturer’s protocol. Single-strand cDNAs were synthesized from 400 ng of total RNA using a ReverTra Ace Kit (TOYOBO Co., Ltd., Tokyo). The quantitative PCR was performed with a THUNDERBIRD SYBR qPCR Mix (TOYOBO Co., Ltd.) in a LightCycler system (Roche Diagnostics Japan, Tokyo). All results from quantitative PCR were calculated relative to 18S rRNA (18S ribosomal RNA) as a normalization control. Primer sequences of the analyzed genes for quantitative PCR are listed in Supplemental Table I.

RNA sequencing (RNA-seq) and transcriptomic analyses: C3H10T1/2 cells were cultured in growth media with or without 10% FBS (fetal bovine serum) for 24 hours and total RNA was collected for transcriptome analysis (RNA-seq). Single-end RNA-seq libraries were prepared using a TruSeq RNA Sample Prep Kit (Illumina). Sequencing runs were performed on an Illumina Genome Analyzer IIX (Illumina, San Diego, CA, USA). Reads of 38bp with a Phred-scaled quality score ≥25 were mapped to the mm9 genome (UCSC genome browser database) using TopHat (v2.0.0) software. After mapping reads, t/r RNA filtering was carried out using BEDtools with...
Figure 3. Pharmacological perturbation of the cell cycle induces collagen production. C3H/10T1/2 cells were treated with double thymidine block (2 mM) or methotrexate (MTX, 10μM, 24 hours). Total RNA or the culture media was analyzed. A: Data show the transcript levels of Ccna2, Ccnb2 and Gmn. B: Data show the transcript levels of Col1a1, Col1a2 and αSMA. C: The collagen content in the culture media was measured colorimetrically. The data represent the mean ± SD. Two-tailed unpaired Welch’s t-test was performed for the statistical analysis. *P < 0.05.
rRNA/rRNA annotations retrieved from the UCSC table browser. Generation of gene expression data, normalization and gene annotation processes were performed using a Genomatix Genome Analyzer (Genomatix, Munich, Germany). For evaluation of gene expression, the normalized expression value (NE-value) was calculated as follows: the number of reads per gene \( \times 10^{10} / \) the number of mapped reads in the genome \( \times \) gene length. Gene ontology enrichment analysis was carried out using DAVID software tools (https://david.ncifcrf.gov/).

**Statistical analysis:** All results are presented as the mean \( \pm \) standard deviation (SD). Two-tailed, unpaired Welch’s \( t \) tests were applied to compare the two groups. Statistical significance was established at \( P < 0.05 \). All calculations were performed using Prism 5 for Windows software (version 5.04; GraphPad, San Diego, CA, USA).

**Results**

**Serum deprivation induces collagen production in C3 H/10T1/2 cells:** To examine whether growth factor or nutrient deficiency affects ECM production, we cultured a fibroblast cell line, C3H/10T1/2 cells, in a serum-deprived condition, and measured the collagen content of the supernatant. Serum deprivation strikingly accelerated collagen production in C3H/10T1/2 cells (Figure 1A, B). The collagen content of the supernatant of serum-deprived fibroblasts was even higher than that of TGF-\( \beta \) stimulated fibroblasts (Figure 1B). We next examined the gene expression levels in fibroblasts. TGF-\( \beta \) significantly increased the transcript levels of type I collagen (Col1a1 and Col1a2), Actin, alpha 2, smooth muscle, aorta (Acta2) and Serine peptidase inhibitor, clade E and member 1 (Serpine 1) (Figure 1C). However, a serum-deprived environment did not affect their transcript levels, indicating that serum deprivation increased collagen production through a mechanism independent of its transcriptional regulation.

**Serum deprivation decreases the transcript levels of cell cycle-related genes:** To understand the roles of serum deprivation on the gene expression profile in C3H/10T1/2 cells, we performed a transcriptome analysis and identified genes whose expressions were affected in a serum-deprived environment (Figure 2A). The transcript levels of 709 genes were decreased (Supplemental Table II), whereas those of 260 genes were upregulated in a serum-deprived condition (Supplemental Table III). We next performed gene ontology enrichment analysis and discovered that genes related to the mitotic nuclear division pathway and the cell cycle pathway are significantly decreased in a serum-deprived condition (Figure 2B). We further confirmed by qPCR that the transcript levels of cell cycle-related genes including Cyclin A2 (Cena2), Cyclin B2 (Ccnb2) and Geminin (Gnnm) are significantly decreased in a serum-starved environment (Figure 2C).

**Cell cycle inhibitors elicit collagen production in C3H/10T1/2 cells:** We next tested whether cell cycle inhibition has a direct role in collagen production, by pharmacologically perturbing the cell cycle in fibroblasts and measuring collagen production. Double thymidine block and MTX significantly decreased the transcript levels of cell cycle-related genes, indicating that these agents perturbed the cell cycle in C3H/10T1/2 cells (Figure 3A). Neither thymidine nor MTX increased the transcript levels of Col1a1, Col1a2 and Acta2 (Figure 3B). Intriguingly, both thymidine and MTX significantly increased collagen abundance in the supernatant of culture media, suggesting that cell cycle perturbation directly facilitates collagen synthesis in a post-transcriptional manner (Figure 3C).

**Discussion**

In this study, we found that a serum-starved environment induces collagen production in C3H/10T1/2 cells. Facilitation of collagen synthesis is mediated through a process independent of the transcriptional levels of type I collagen genes. Pharmacological experiments showed that cell cycle perturbation directly accelerates collagen synthesis in fibroblasts.

While the results uncovered a link between the cell cycle and fibroblast activation that was previously not described, it remains unclear how cell cycle arrest induces collagen production in fibroblasts. As energy substrate utilization is known to affect cellular function,\(^5\) it may account for the acceleration of collagen production in cell cycle-arrested fibroblasts. Glycine is the most abundant amino acid in the collagen peptide chain. Importantly, glycine is also used for the biosynthesis of purine nucleotides, which are essential for the proliferation of C3H/10T1/2 cells. Therefore, we speculate that collagen synthesis and cell proliferation may act antagonistically with regard to the utilization of glycine and potentially other energy substrates. Inhibition of the cell cycle may redirect the intracellular use of glycine away from cell proliferation and towards collagen synthesis.

MTX induces cell cycle arrest and is clinically used to treat patients with rheumatoid arthritis. Pulmonary fibrosis is one of the adverse effects observed in patients treated with MTX.\(^13\) In addition, several antineoplastic agents, including bleomycin and irinotecan, or radiation therapy can elicit pulmonary fibrosis.\(^14\-16\) The results in this study illuminate a previously undescribed pathway which may have implications for chemotherapy or radiation-induced organ fibrosis.

Management of excessive tissue fibrosis is crucial to maintain the homeostasis of the organs. In order to establish anti-fibrotic therapy for heart diseases, we need to target the malignant excessive fibrosis specifically, while leaving the reparative replacement fibrosis unaffected. Exploring the molecular processes in fibroblast activation will allow us to better understand the roles of tissue fibrosis in organ integrity.

**Disclosures**

**Conflicts of interest:** None.
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Supplemental Files

Supplemental Tables I-III
Please see supplemental files; https://doi.org/10.1536/ihj.18-710