Absence of Type VI Collagen Paradoxically Improves Cardiac Function, Structure, and Remodeling After Myocardial Infarction

Daniel J. Luther, Charles K. Thodeti, Patricia E. Shamhart, Ravi K. Adapala, Cheryl Hodnichak, Dorothee Weihrauch, Paolo Bonaldo, William M. Chilian, J. Gary Meszaros

Rationale: We previously reported that type VI collagen deposition increases in the infarcted myocardium in vivo. To date, a specific role for this nonfibrillar collagen has not been explored in the setting of myocardial-infarction (MI).

Objective: To determine whether deletion of type VI collagen in an in vivo model of wound healing after MI would alter cardiac function and remodeling in the days to weeks after injury.

Methods and Results: Wild-type and Col6a1−/− mice were subjected to MI, followed by serial echocardiographic and histological assessments. At 8 weeks after MI, infarct size was significantly reduced, ejection fraction was significantly preserved (43.9% ±3.3% versus 29.1% ±4.3% for wild-type), and left ventricular chamber dilation was attenuated in the Col6a1−/− MI group (25.8% ±7.9% increase versus 62.6% ±16.5% for wild-type). The improvement in cardiac remodeling was evident as early as 10 days after MI in the Col6a1−/− mice. Myocyte apoptosis within the infarcted zones was initially greater in the Col6a1−/− group 3 days after MI, but by day 14 this was significantly reduced. Collagen deposition also was reduced in the infarcted and remote areas of the Col6a1−/− hearts. The reductions in chronic myocyte apoptosis and fibrosis are critical events leading to improved long-term remodeling and functional outcomes.

Conclusions: These unexpected results demonstrate for the first time to our knowledge that deletion of type VI collagen in this knockout model plays a critical protective role after MI by limiting infarct size, chronic apoptosis, aberrant remodeling, and fibrosis, leading to preservation of cardiac function. (Circ Res. 2012; 110:00-00.)

Key Words: cardioprotection □ cell matrix □ interactions □ myocardial infarction remodeling □ myofibroblast □ nonfibrillar collagen

The extracellular matrix plays a key role in cardiac remodeling and wound repair after a myocardial infarction (MI). Patients who survive a MI normally have development of cardiac fibrosis, which contributes to the decline in cardiac function and eventual failure. Matrix turnover is critical in the days and weeks after MI; however, the functions of specific extracellular matrix components in this process remain poorly defined.

It has been accepted that type I and type III collagen are major constituents of the cardiac extracellular matrix that provide structural and mechanical support to the heart and act as signaling conduits between myocardial cells. Whereas the extracellular matrix field has focused on these fibrillar collagens, our recent studies have demonstrated that type VI collagen induces myofibroblast differentiation in vitro and that its deposition is enhanced in vivo after MI.

Type VI collagen mutations can cause Bethlem myopathy, an age-related disease characterized by skeletal muscle weakness and limited life span. Type VI collagen is a nonfibrillar collagen that assembles end-to-end in a beaded filament arrangement. Typically interspersed with types I and III collagen, collagen VI forms a microfibril network that organizes the fibrillar collagens and anchors...
these to the basement membrane.9 A model for Bethlem myopathy was generated by targeted deletion of the *Col6a1* gene, which causes early-onset myopathy.10,11 Although skeletal muscle defects caused by collagen type VI mutations have been described, none has determined the consequences of its absence using MI injury models.12,13 As well as in other tissues such as adipose, cartilage, brain, and tendon.14–16 Here, we demonstrate that the absence of type VI collagen provides a profound beneficial effect on cardiac function and remodeling after MI.

Materials and Methods

**Animal Model**

Seventy-five male *Col6a1*−/− and wild-type (WT) mice in the CD-1 background at 12 to 16 weeks of age were used in this study. Detailed Methods are provided in the Online Supplement.

**Surgical Induction of MI**

Mice were anesthetized by intraperitoneal injection of sodium pentobarbital (70 mg/kg), and the heart was accessed via left thoracotomy and MI was induced by permanent occlusion of the left anterior descending artery.

**Histological Assessment of Apoptosis, Fibrosis, and Infarct Size**

Animals were euthanized and cryosections were prepared for apoptosis assessment using TUNEL staining method as previously described.6,7 Additional hearts were embedded in paraffin and entire hearts were sectioned serially for assessment of collagen deposition using Masson trichrome, picrosirius red, and picrosirius red under polarized light. Infarct sizes were quantitated by 2,3,5-triphenyltetrazolium chloride staining.

**Echocardiographic Assessment**

Two-dimensional echocardiography was performed and calculations were performed offline by double-blinded reviewers using the Vevo 770/3.0 system and software (VisualSonics).

**Statistical Analysis**

Data analysis was performed using Graphpad Prism 4.0 software (Graphpad Software). Significance was determined by ANOVA with Bonferroni post-test (*P*<0.05 considered significant).

**Results**

**Physiological Measurements of *Col6a1*−/− Versus WT Mice Before and After MI**

The physiological features of WT and *Col6a1*−/− mice are outlined in Table. *Col6a1*−/− mice are consistently smaller than WT mice, and heart weights were lower in *Col6a1*−/− mice in sham and MI groups when compared to WT. After MI the ejection fraction, fractional shortening and cardiac index were higher in *Col6a1*−/− hearts, whereas left ventricular (LV) mass and wall thinning were reduced.

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<th>Table. Echocardiographic Assessment of Wild-Type and <em>Col6a1</em>−/− Mice 8 Weeks After Myocardial Infarction</th>
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BW indicates body weight; CI, cardiac index; DT, deceleration time; FAC, fractional area change; HR, heart rate; HW, heart weight; LVIDd, left ventricular internal diameter, diastole; LVIDds, left ventricular internal diameter, systole; MV, mitral valve; RWT, relative wall thickness.

*P*<0.05 vs WT sham.

†*P*<0.05 vs *Col6a1*−/− sham (n=8/group).

‡*P*<0.05 vs WT MI.
WT at 8 weeks after MI (0.25/H11006 these ratios were diminished in the knockouts compared to
29.42/4.05; Figure 2B; /H11006 Doppler echocardiography on mice 3 days to 8 weeks after
Cardiac function was assessed using 2-dimensional and
Col6a1 Improved Cardiac Function and LV Dimensions in
Col6a1 (percent collagen/LV area) was significantly reduced in
picrosirius red staining (Figure 2A). Collagen volume
infarcted zones was visualized by Masson trichrome and
Col6a1 Long-Term Myocyte/Nonmyocyte Apoptosis in the
Reduced Collagen Volume and Decreased
Figure 1. Cardiac structure and function are preserved in Col6−/− mice after myocardial infarction (MI). A and B, Representative images taken at 8 weeks after MI demonstrate preservation of left ventricular (LV) wall integrity, greater wall kinesis, and reduced chamber size in Col6−/− hearts (n=8). C and D, 2,3,5-tri-phenyltetrazolium chloride (TTC) staining performed on hearts 3 days after MI revealed no significant differences in Inf/LV area ratio in Col6−/− versus wild-type (WT) mice; however, infarct size at 8 weeks is significantly greater in WT (n=4/group). P<0.05.

(Figure 1A). Gross observation and measurement of 2-dimensional guided M-mode tracings show improved wall thickness, chamber dimension, and anterior wall kinesis in Col6α1−/− MI mice (Figure 1B), illustrating the preserved myocardial function in the knockout hearts. Area at risk percentage (Supplemental Figure 1) and infarct/LV area ratios were not significantly different in the Col6α1−/− mice versus WT 3 days after MI; however, these ratios were diminished in the knockouts compared to WT at 8 weeks after MI (0.25±0.01 versus 0.37±0.05, respectively; Figure 1C, D).

Reduced Collagen Volume and Decreased
Long-Term Myocyte/Nonmyocyte Apoptosis in the
Col6α1−/− Hearts
Quantitative analysis of total collagen content in the LV and infarcted zones was visualized by Masson trichrome and picrosirius red staining (Figure 2A). Collagen volume (percent collagen/LV area) was significantly reduced in Col6α1−/− MI mice compared to WT (15.89±0.84 versus 29.42±4.05; Figure 2B; P<0.05). Collagen levels of WT and Col6α1−/− sham mice were not significantly different (data not shown).

TUNEL staining revealed an initial increase in apoptosis within the infarcted area of the Col6α1−/− hearts at 3 days (acute phase), followed by a reduction by 14 days (chronic phase) relative to WT hearts (Figure 2C, D; P<0.05). Importantly, the ratio of myocyte/nonmyocyte apoptosis by day 14 was significantly decreased in the knockout hearts compared to WT (Figure 2E).

Improved Cardiac Function and LV Dimensions in
Col6α1−/− Mice 10 Days to 8 Weeks After MI
Cardiac function was assessed using 2-dimensional and Doppler echocardiography on mice 3 days to 8 weeks after MI. Echocardiography at 3 days (Figure 3A) revealed that cardiac function is not significantly different between the null and WT MI mice. Differences in LV diastolic volume were also not apparent at this early time (Figure 3B). However, serial measurements of function and remodeling revealed that in Col6α1−/− mice, ejection fraction was preserved as early as 3 weeks and persisted to 8 weeks after MI (Figure 3C, E; 43.9%±3.3% versus 29.1%±4.3%; P<0.05) and LV chamber volume was reduced beginning at 10 days after MI (Figure 3D, F; 25.8%±7.9% versus 62.6%±16.5% increase in LV volume over shams; P<0.05). Cardiac index increased in Col6α1−/− mice after MI versus WT (0.55±0.02 versus 0.48±0.04; P<0.05; Table).

Discussion
These data are the first to our knowledge to demonstrate that the lack of collagen VI significantly and paradoxically improves remodeling after MI in response to permanent left anterior descending artery occlusion. We originally predicted that the collagen VI-deficient mice would experience deficits in remodeling, because this collagen has been proposed to play a critical role in organizing and anchoring the fibrillar type I and III collagen network.9,17 This, along with the information taken from other tissues (skin, tendon, skeletal muscle), and collagen knockouts have all shaped our original hypothesis that deletion of collagen VI would result in a loss of function.

Elegant extracellular matrix studies uncovered the function of collagen VI to organize the fibrillar collagen (mainly type I) network in skin.9 Izu et al16 recently reported significantly decreased collagen diameter and compromised mechanical properties in tendons of the Col6α1−/− mice. Another intriguing study demonstrated that collagen knockout null mice with experimentally induced hypertension had a poorly organized
fibrillar collagen network, impaired microvascular hemodynamics, and irregularly organized cardiac myocytes.\textsuperscript{18} Our results are particularly “paradoxical” because of the known skeletal muscle phenotype of this knockout model originally reported as a model of Bethlem Myopathy by Bonaldo et al.\textsuperscript{10} This seminal study was followed by several subsequent reports indicating that the skeletal myocytes had premature mitochondrial transition pore opening and apoptosis leading to skeletal muscle dystrophy as the mice aged beyond 32 weeks.\textsuperscript{12} Prevention of mitochondrial transitional pore opening has been shown to be cardioprotective,\textsuperscript{19} indicating that this may be a common target for prevention of skeletal and cardiac myocyte dysfunction and death. It is important to note in our study that we utilized young mice for our MI studies (12–16 weeks), an age at which there were no differences in baseline cardiac function and no outward signs of skeletal muscle weakness. It is of interest to perform aging studies on the \textit{Col6a1}\textsuperscript{-/-} mice to determine if the cardioprotection seen in the current study persists after the onset of Bethlem myopathy.

The improvement in remodeling after MI in the \textit{Col6a1}\textsuperscript{-/-} hearts is, at least in part, likely because of accelerated remodeling and apoptosis followed by a reduction in chronic apoptosis. The increased apoptosis at 3 days after MI in the knockouts suggests that injury responses began earlier in these animals, which appears to be beneficial to the long-term outcomes. Our data also indicate that less myocyte apoptosis occurs in the knockouts after 14 days, which supports the notion that wound healing occurs, and is completed, earlier in the \textit{Col6a1}\textsuperscript{-/-} mice. Furthermore, the more chronic apoptosis evident at 14 days after MI in the WT mice may contribute to the increased scarring and fibrosis, followed by long-term loss of cardiac function. Other cardioprotective mechanisms may exist as well, because the structure–function relationships relating to collagen VI and the fibrillar collagens have not been established in the myocardium. The previous studies of skin\textsuperscript{9} and tendon\textsuperscript{16} (discussed) suggest that the absence of collagen VI affects fibrillar collagen network organization, which, in our case, could be creating a more biomechanically advantageous environment for wound healing after MI. Alternatively, the possibility exists that cardioprotection may involve changes in mitochondrial function;\textsuperscript{19} however, mitochondrial function is altered and induces apoptosis in the skeletal muscle of \textit{Col6a1}\textsuperscript{-/-} mice.\textsuperscript{12} The mechanisms responsible for these contrasting phenotypes in cardiac and skeletal muscle are not known and require further investigation.

In conclusion, the confluence of previous reports and our current findings demonstrate a critical and novel role for type VI collagen in myocardial injury and remodeling. Importantly, our study is the first to our knowledge to describe the
cardioprotective effects of collagen VI deletion to enhance cardiac function and limit aberrant remodeling after MI, and it identifies a potentially novel target to treat postischemic injury in the myocardium.

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Disclosures
None.

References
Novelty and Significance

What Is Known?

● Collagen VI organizes and anchors the fibrillar collagen network in many tissues, and its deficiency causes age-related defects in skeletal muscle function.

● Postinfarction remodeling depends on collagen and extracellular matrix synthesis to stabilize the scar and improve long-term remodeling.

● The prevailing idea is that deposition of fibrillar collagens type I and III are increased after myocardial infarction (MI) and are the key mediators of infarct scar assembly.

● Type VI collagen deposition increases after MI; however, the importance of this nonfibrillar collagen in remodeling after MI is unknown.

What New Information Does This Article Contribute?

● Knockout of type VI collagen improves remodeling after MI by limiting infarct size, collagen deposition, and chronic myocyte apoptosis.

● Absence of collagen VI preserves long-term cardiac performance after MI, and it prevents left ventricular (LV) wall thinning and limits LV chamber dilation.

● The beneficial effects of collagen VI deletion suggest that this nonfibrillar collagen plays a key role in wound healing and remodeling after MI.

Type VI collagen is a nonfibrillar filamentous collagen produced by activated fibroblasts that plays roles in fibrillogenesis and organization and anchoring of fibrillar collagens. Our goal was to determine whether collagen VI contributes significantly to wound healing after MI by using a relevant in vivo MI model (collagen VI-deficient mice). This study is the first to our knowledge to report an unexpected and novel cardioprotective effect of collagen VI deletion in preserving cardiac structure and preventing pathological remodeling after MI. These findings may provide the basis for the development of collagen-based therapies to limit adverse remodeling after MI.
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Supplemental Material

**Animals**

Male Col6a1<sup>−/−</sup> and WT mice in the CD-1 background (13) and at the ages of 12-16 weeks of age were used in this study (n=75; MI=47, sham=28). The mice were housed individually in a temperature- and humidity-controlled environment (20 ± 2 °C and 55 ± 10% relative humidity) and kept on a 12:12 h day-night cycle access to standard rat chow (Purina, Copley Feed, Copley, OH) and tap water ad libitum. All studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the Northeast Ohio Medical University, Rootstown, OH (Protocol # 08-019).

**Surgical protocol**

Mice were anesthetized by i.p. injection of sodium pentobarbital (70 mg/kg). Mice were moved to a surgical platform and body temperature maintained at 37 ± 1 °C. Mice were ventilated via tracheal intubation connected to a rodent ventilator (Minivent type 845; Harvard Apparatus). A left thoracotomy was performed through the fourth intercostal space and occlusion of the left anterior descending artery (LAD) was performed using 8-0 nylon suture. ST segment elevation was used to confirm MI induction. The thoracic cavity was evacuated via chest tube and closed using 5-0 Vicryl suture. The mice were moved to a heated recovery area and supplemented with 100% oxygen. Sham operations were performed on animals by passing suture beneath the LAD without ligating the vessel.

**Histological assessment of apoptosis, fibrosis and myocyte size**

Animals were euthanized at the indicated time points after infarction. Following whole animal perfusions of PBS and 4% paraformaldehyde (PFA), hearts were rinsed in ice cold PBS and incubated overnight at 4°C in 4% PFA. For hearts dedicated to TUNEL staining, samples were rinsed and then incubated overnight in 7% sucrose and frozen in Tissue-Tek tissue-freezing medium (Miles; Elkhart, IN). Transverse cryosections were cut including both infarcted and non-infarcted regions at a thickness of 7 µm on a Leica cryostat and placed on super frost plus coated slides. TUNEL staining was performed on 3 and 14 day post-MI hearts from each group (WT, Col6a1<sup>−/−</sup>) and the number of TUNEL positive cells per field (Figure 2D) were determined from 3 hearts per group with 5 fields counted for each under 10x (fields contained 200-250 cells each) by confocal microscopy. Cardiac myocyte and non-myocytes were differentiated by their localization within the myocardial tissue or interstitial space under enhanced 40x confocal imaging, with serial sections from each stained with beta-dystroglycan to visualize the cell membranes and confirm the localization of the staining as interstitial or intramuscular.

For collagen assessment, hearts were excised and incubated overnight in PFA, embedded in paraffin and sectioned serially at a thickness of 7µm using a microtome. Transverse sections were stained consecutively using Masson’s trichrome and PSR, followed by bright field imaging and polarized light microscopy. A 2X objective was used to image sections from 6 infarcted planes of each heart that were then overlaid with a 35x40 grid. Point counting was used to determine percent volume faction of collagen (C<sub>VF</sub>) using the equation C<sub>VF</sub> = (P<sub>C</sub>/P<sub>R</sub>) x 100 % (where P<sub>C</sub> equals the number of intersections with collagen and P<sub>R</sub> the total number of intersections in the entire sample area).4

Myocyte size was measured using Masson’s trichrome stained heart transverse sections to visualize myocyte cross-sections. Diameter measurements were made across cells with visible nuclei and quantitated using Metamorph Image Analysis software (SPOT Imaging Solutions, Sterling Hts., MI) (n=150 cells/group; 4 animals/group).
Assessment of infarct size

Infarct size was assessed using 2,3,5-triphenyltetrazolium chloride (TTC) method at 3 day post-MI. Intact hearts were cannulated and retrograde perfused with 2% Evan’s blue stain to map area at risk (AAR) region. Hearts were then isolated and sectioned into 2 mm transverse slices and incubated in TTC at 37°C for 20 minutes to allow for differentiation between viable (red) and necrotic (white) tissues and incubated in 4% PFA for 20 minutes at room temperature. Sections were then imaged and assessed by planimetry method using ImageJ Software (NIH, Bethesda, MD).

Echocardiography

In vivo heart function was assessed using a Vevo 770 system (VisualSonics, Inc., Toronto, Ontario, Canada) with a 710B-075 transducer (20-30 MHz) designed specifically for small animal studies at a frame rate of 40-60 Hz. Mice were anesthetized using 2-2.5% sevoflurane via nose cone, placed on an adjustable platform equipped with ECG electrodes to monitor heart and respiration rates. Body temperature was maintained at 37.0 ± 1°C and two-dimensional (2D), M mode, and pulsed-wave (PW) Doppler echocardiography images were obtained in parasternal short-, long-axis, apical and four chamber views by previously validated techniques (1-3). M-mode and 2D images at the mid-papillary level were obtained from the parasternal short axis view. Mitral valve (MV) inflow was obtained from apical four-chamber view followed by PW Doppler interrogation of MV inflow at the tips of MV leaflets. A complete study with image acquisition was carried out in approximately 10 min. All presented measurements were averaged from at least three cardiac cycles. Calculations and measurements were carried out offline by blinded reviewers using the Vevo 770/3.0 software.

Statistical analysis

Data analysis was performed using Graphpad Prism 4.0 statistical analysis software (Graphpad Software, La Jolla, CA). Statistical significance was determined by ANOVA with Bonferroni’s multiple comparison test.

References (Supplemental Materials)

Online Figure I. (A and B) Evan’s Blue and TTC staining performed on 3 day post-MI hearts reveals no significant differences in Inf/AAR ratio (C) and %AAR (D) in Col6⁻/⁻ versus WT mice indicating that the ischemic injuries are similar in size after induction of MI (n=5).
Online Figure II. Baseline control hearts and 8 week post-MI were cross-sectioned and stained to clearly visualize and measure individual myocytes. Comparison of myocyte cross-sectional diameter in control animals revealed no difference in myocyte size; however, at 8 weeks post-MI data revealed myocyte hypertrophy in WT mice (n=150/group; 4 mice/group) *p<0.05.