Circulation Research
JOURNAL OF THE AMERICAN HEART ASSOCIATION

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Circ. Res. 2004;95;253-260; originally published online Jun 24, 2004;
DOI: 10.1161/01.RES.0000136520.07995.aa
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Valvular Myofibroblast Activation by Transforming Growth Factor-β

Implications for Pathological Extracellular Matrix Remodeling in Heart Valve Disease

Gennyne A. Walker, Kristyn S. Masters, Darshita N. Shah, Kristi S. Anseth, Leslie A. Leinwand

***Abstract*—**The pathogenesis of cardiac valve disease correlates with the emergence of muscle-like fibroblasts (myofibroblasts). These cells display prominent stress fibers containing α-smooth muscle actin (α-SMA) and are believed to differentiate from valvular interstitial cells (VICs). However, the biological factors that initiate myofibroblast differentiation and activation in valves remain unidentified. We show that transforming growth factor-β1 (TGF-β1) mediates differentiation of VICs into active myofibroblasts in vitro in a dose-dependent manner, as determined by a significant increase in α-SMA and the dramatic augmentation of stress fiber formation and alignment. Additionally, TGF-β1 and increased mechanical stress function synergistically to enhance contractility. In turn, contractile valve myofibroblasts exert tension on the extracellular matrix, resulting in a dramatic realignment of extracellular fibronectin fibrils. TGF-β1 also inhibits valve myofibroblast proliferation without enhancing apoptosis. Our results are consistent with activation of a highly contractile myofibroblast phenotype by TGF-β1 and are the first to connect valve myofibroblast contractility with pathological valve matrix remodeling. We suggest that the activation of contractile myofibroblasts by TGF-β1 may be a significant first step in promoting alterations to the valve matrix architecture that are evident in valvular heart disease. **(*Circ Res*. 2004;95:253-260.)**

Key Words: valvular heart disease ■ valvular interstitial cells ■ myofibroblasts ■ TGF-β1 ■ α-smooth muscle actin ■ contractility

Cardiac valves are connective tissue structures whose function is necessary for proper hemodynamic flow. Valvular interstitial cells (VICs) are the most prevalent cells of heart valve leaflets and are responsible for maintaining the structural integrity of the valve.[1–3] In addition to a cellular component, valve leaflets are comprised of extracellular matrix (ECM) proteins, primarily collagen, elastin, and glycosoaminoglycans, arranged exquisitely to achieve a structure that is flexible enough to change shape easily, yet strong enough to withstand elevated hemodynamic stress.[4] Valve structure maintenance requires proper ECM metabolism, a function attributed to VICs. These spindle-shaped mesenchymal cells secrete ECM components including collagen, fibronectin, chondroitin sulfate, and prolyl-4-hydroxlase.[5] They also express ECM-degrading enzymes such as matrix metalloproteases (MMPs)[6,7] and cathespin D.[8] In addition to their role in healthy valves, recent histochemical and molecular data have indicated that VICs are actually the precursors of valve myofibroblasts, muscle-like cells that are observed in diseased heart valves.[5,9–11] Currently, little information exists regarding the molecular details of the VIC myofibroblastic phenotype, the conditions under which this phenotype may be activated, and the role myofibroblasts play in valve disease.

Myofibroblasts are "hyperactivated" fibroblasts with properties of fibroblasts and muscle cells[12–15] that facilitate tissue remodeling and wound healing but also play a pathological role in fibrotic disease. Compared with their precursor cell type (referred to as stellate transformed[16] or protomyofibroblasts[12]), activated myofibroblasts have dramatically higher levels of ECM and cytokine secretion, increased contraction,[14] and a trademark stellate morphology with prominent stress fibers. Histochemically, myofibroblasts are identified by the expression of α-smooth muscle actin (α-SMA).[17,18] However, they can also express other contractile proteins, such as the striated-muscle isoforms of myosin heavy chain (MyHC).[13,19,20] Myofibroblast activation is regulated tightly by cytokines that control differentiation, proliferation, contraction, ECM secretion, and migration to the site of wound healing or tissue remodeling.[14] After completion of remodeling activities, myofibroblasts are eliminated by apoptosis;[21] however, when the myofibroblast life cycle is not regulated

Original received July 28, 2003; resubmission received December 15, 2003; revised resubmission received June 11, 2004; accepted June 11, 2004.

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Circulation Research is available at http://www.circresaha.org DOI: 10.1161/01.RES.0000136520.07995.aa

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properly, myofibroblasts persist with continued force generation and ECM production, resulting in pathological fibrosis, scarring, and fibrocontractile disease.[22]

An indisputable role for myofibroblasts in valvular wound healing and pathology is illustrated by immunohistological comparisons of normal, wounded, and diseased valves. In healthy valves, the majority of the VIC population expresses little if any α-SMA.[5,10,23] Increased numbers of α-SMA–positive VICs (activated valve myofibroblasts) are seen in valves that have been mechanically injured,[24–26] affected by myxomatous degeneration[10] or endocarditis,[6] and in valves exposed to elevated blood serotonin levels (carcinoid syndrome).[9] The appearance and localization of myofibroblasts in valve tissue correlate strongly with matrix disarray, degenerative lesions, increased MMP mRNA levels, and fibrosis,[6,10,27,28] implicating their role in valve disease. Although recent reports have identified factors that stimulate ECM synthesis by activated valve myofibroblasts, factors that regulate their contractile properties are poorly understood. We believe characterizing the mechanisms that activate valve myofibroblast contractility is critical to understanding the progression of valve pathologies. Additionally, information regarding these mechanisms is applicable particularly to cardiovascular tissue engineering, where a better understanding of valve tissue morphogenesis will lead to more innovative protocols for engineering valve tissue equivalents.[29]

Little is still known about VIC biology or the elements that activate the valve myofibroblast phenotype. Valve tissue responds to injury in a manner similar to the other tissues, in which the healing response is initiated by elevated levels of transforming growth factor-β (TGF-β).[30] Although TGF-β2 and TGF-β3 isoforms have distinct functions in the epithelial–mesenchymal transformation that occurs during cardiac valve development,[31] TGF-β1 appears to play a greater role in adult valve remodeling and pathology. TGF-β1 is known for its ability to differentiate mesenchymal cells into myofibroblasts and to regulate multiple aspects of the myofibroblast phenotype through transcriptional activation of α-SMA, collagen, MMPs, and other cytokines.[32] Elevated TGF-β1 levels correlate with increased matrix production at sites of pathological fibrosis, where activated myofibroblasts persist and produce high levels of ECM components.[33] With respect to VIC biology, heart valves subject to carcinoid syndrome exhibit increased expression of TGF-β1 and TGF-β3[34] that correlates with α-SMA–positive fibroblasts, increased collagen deposition, changes in the amount and organization of ECM components,[9] and calcification.[36,60] Persistence of α-SMA–positive fibroblasts in diseased valves suggests to us that TGF-β1 plays an active role in altering the biological activity of VICs by activating the myofibroblast phenotype and regulating contraction of these cells.

Given that valve disease is associated with alterations in the biological activity of VICs, we sought to explore further the molecular response of VICs to TGF-β1. We demonstrate that TGF-β1 is a key activator of the valve myofibroblast phenotype: TGF-β1 increases α-SMA expression, promotes stress fiber formation, and inhibits proliferation without increased apoptosis. Coincident with elevated α-SMA expression, TGF-β1 enhances the ability of VICs to exert force on an anchored collagen substrate and promotes realignment of extracellular fibronectin fibrils. These results allow us to propose that elevated TGF-β1 concentrations in vivo induce valve myofibroblast contraction, enhance their responsiveness to mechanical stress, and promote their persistence in valve disease.

Materials and Methods

Cell Culture

VICs were isolated from porcine aortic valves by collagenase digestion[35] and cultured in growth media (Media 199, 15% FBS, 2 mmol/L glutamine, and 100 U/mL penicillin/streptomycin) on tissue culture plastic for 3 to 5 passages. TGF-β1 (Sigma) was used at 5, 0.5, and 0.05 ng/mL. Unless otherwise stated, cell confluence did not exceed 70%. TGF-β–neutralizing antibody (pan-anti-TGF-β; R&D Systems) was added at 50 μg/mL.

Immunocytochemistry

VICs seeded on glass coverslips were cultured for 48 hours, then fixed, permeabilized, and incubated with the antibodies listed in supplemental Table I (available online at http://circres.ahajournals.org). Specific staining was detected using either a fluorescein isothiocyanate–conjugated anti-mouse antibody or a Texas Red–conjugated anti-rabbit antibody (The Jackson Laboratory).

Immunoblots

VICs were incubated for 5 days in growth media supplemented with TGF-β1 or neutralizing antibody and passaged every 2 days to avoid the nodule-forming phenomena that promotes cell death.[36,37] Cells were lysed with lysis buffer (10 mmol/L NaCl, 10 mmol/L Tris-HCl, pH 8.0, 3 mmol/L $MgCl_2$, and 0.5% Nonidet P-40). Total protein was separated by SDS-PAGE. Goat anti-mouse antibody conjugated to an infrared dye (IgG-IR dye 800; Rockland) was used to visualize specific bands. Membranes were scanned on a Li-Cor infrared imaging system using Odyssey software (Li-Cor). Results are the sum of 3 experiments.

Contraction Assays

The contractile response of VICs to TGF-β1 was evaluated in collagen matrices.[19,38] Matrices were formed by seeding 16-mm wells (24-well culture plate) with 700 μL of a collagen–VIC mixture: 1.9 mg/mL of collagen (Vitrogen 100; Cohesion Technologies), 1×10^5 cells/mL, and TGF-β1. Floating matrices were detached after 1 hour of polymerization. Stressed matrices were kept adhered to the well for 120 hours before release. Cytochalasin D (Sigma) was added 30 minutes before matrix release. Diameters were measured over time. Experiments were performed in triplicate, 3×.

Promoter Analysis

The rat α-SMA upstream regulatory region was cloned into pGL3-Basic (Promega) upstream of the firefly luciferase gene.[20] VICs were transfected using Lipofectamine 2000 (Invitrogen). Luciferase activity was assayed using the Promega luciferase assay system. Each experiment was performed in quadruplicate, twice. Results are expressed as absolute light units, normalized to the untreated control.

DNA Synthesis

The effect of TGF-β1 on VIC DNA synthesis was measured by $[^3H]$-thymidine incorporation in serum-stimulated VIC cultures.[35] Results are reported as the percentage of $[^3H]$ incorporation compared with the untreated control. The experiment was performed twice, in quadruplicate.

Apoptosis

Apoptosis was assessed with the APOPercentage assay kit (Accurate Chemicals). VICs were seeded at a concentration of 2×10^4 cells/cm^2 in gelatin-coated wells, incubated with TGF-β for 7 and 48 hours,

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Analysis of Muscle Gene Expression in Porcine Aortic VICs

Myofibroblast markers	
α-SMA	++
Desmin	±
Vimentin	+++
Smooth muscle MyHC	−
Sarcomeric proteins	
Sarcomeric MyHC	−
Sarcomeric troponin T	−
Muscle transcription factors	
Myf-5	±*
MyoD	−
Myogenin	−
MRF4	ND

The expression of myofibroblast- and muscle-specific proteins was assessed in VICs. The percentage of positive cells was calculated as the No. of immunoreactive cells divided by the total No. of nuclei within a field. Ten fields were assayed and rated as follows: −, 0%; ±, <5%; +, 5% to 40%; ++, 40% to 80%; +++, >80%.

*Myf-5 detection varied between isolations (see 'Discussion').

MyoD indicates myogenic determination factor; MRF4, muscle regulatory factor 4; ND, not determined.

with 5% ethanol (positive control) for 1 hour, or left untreated (negative control). APOPercentage dye was added to the wells at 3.75 μmol/L for 1 hour at 37°C. Wells were washed twice with PBS, and the dye was solubilized in 0.1 NaOH. Absorbance values were read at 544 nm on a microplate colorimeter and quantified relative to the untreated control. The experiment was performed twice, in quadruplicate.

Statistical Analysis

Statistical significance was determined using a 2-sample t test, assuming unequal variances. SE was calculated as the SD divided by the square root of n, the number of replicates. Results were significant compared with the untreated control if $P<0.05$.

Results

α-SMA Is the Predominant Contractile Protein Expressed in Porcine Aortic VICs

Inherent to the myofibroblast phenotype is the expression of muscle-specific cytoskeletal proteins, including α-SMA and desmin, most often in the absence of smooth muscle myosin. Sarcomeric MyHC isoforms of striated muscle may also distinguish myofibroblasts from smooth muscle cells[13,19] and can contribute to myofibroblast contraction.[20] To better characterize the muscle–gene expression program in cultured VICs, we performed indirect immunofluorescence with a variety of antibodies directed against muscle proteins (Table). In agreement with other studies,[5,39] we found that when cultured on plastic (a more rigid substrate than collagen), 50% to 65% of VICs isolated from porcine aortic valves expressed α-SMA that localized to well-formed, longitudinal stress fibers (Figure 1A). More than 95% of VICs expressed the intermediate filament vimentin, but only rarely (≈1%) was a desmin-positive cell observed. We were unable to detect sarcomeric MyHC by either immunofluorescence with a pan-sarcomeric MyHC antibody (F59) or RT-PCR (data not shown) with a sarcomeric MyHC consensus primer set.[40] We also were unable to detect other sarcomeric muscle-specific proteins such as cardiac troponin T (Table). These results conflict with Roy et al,[41] who detected sarcomeric MyHC mRNA in human VICs using RT-PCR. In conclusion, the only contractile protein detected was α-SMA, an actin isoform whose capacity to confer enhanced contractile function has been demonstrated.[42]

TGF-β1 Activation of Valvular Myofibroblasts

TGF-β1 is an integral activator of the myofibroblast phenotype during wound healing processes.[43] This phenotypic change is marked by the de novo induction of α-SMA expression and its localization to stress fibers.[18] Although interstitial cells within the intact valve cusp express only minimal amounts of α-SMA,[5,10,23] a large percentage of VICs in culture exhibit the activated, α-SMA–positive myofibroblast phenotype,[5,11] likely the result of culture on plastic, a more rigid medium.[14] To determine whether autocrine TGF-β secretion participates in valve myofibroblast activation in vitro, VICs were incubated for 5 days with a neutralizing antibody that recognizes the major TGF-β isoforms. TGF-β neutralization significantly decreased the level of α-SMA, as assayed by immunofluorescence and immunoblot (Figure 1C and 1D), demonstrating that cultured VICs secrete TGF-β, which induces the α-SMA–positive phenotype. Conversely, increasing the concentration of free TGF-β1 in culture induced the α-SMA–positive myofibroblast phenotype. Immunoblot analysis demonstrates that α-SMA expression is increased 230%±30% at the highest concentration of TGF-β1 tested (5 ng/mL), a measurement that had not been reported previously. Modulation of α-SMA expression by TGF-β1 occurs at least in part through direct activation of α-SMA promoter activity (Figure 1E), where a strong correlation between α-SMA promoter activation and protein levels in response to TGF-β1 is observed.

Activated Valve Myofibroblasts Alter Extracellular Fibronectin Organization

TGF-β1 not only increases the amount of α-SMA protein expressed but also promotes development of α-SMA–positive stress fibers (Figure 2, α-SMA panels). When VICs are cultured at high density in the presence of TGF-β1, they begin to aggregate, calcify, and die.[37] This phenomenon is prevented by actin polymerization with cytochalasin D,[36] indicating that the actin cytoskeleton is required for this process. By following α-SMA expression for 48 hours (Figure 2, phase panels), we observed that the rate and extent of this process is dose-dependent and involves the striking formation and arrangement of α-SMA–positive stress fibers aligned perpendicular to the developing ridges. In fact, the α-SMA–containing cytoskeleton appears to be pulling or straining against the rigid plastic surface, away from ridge formation. Ultimately, cells detach and migrate into a developing nodule (Figure 2, α-SMA panels).

Stress fibers form to counteract elevated external mechanical tension.[44,45] When stress fibers are stimulated to contract, the resulting forces are transmitted from the actin cytoskeleton to the ECM through fibronectin-containing focal adhe-

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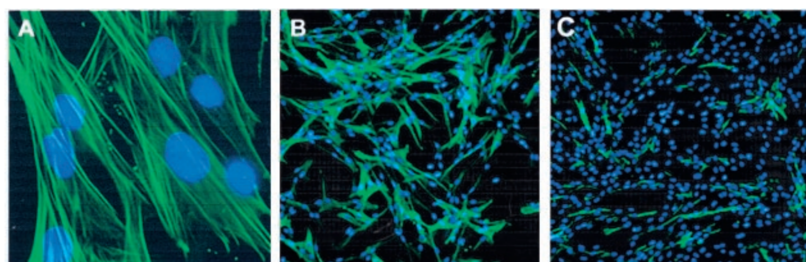

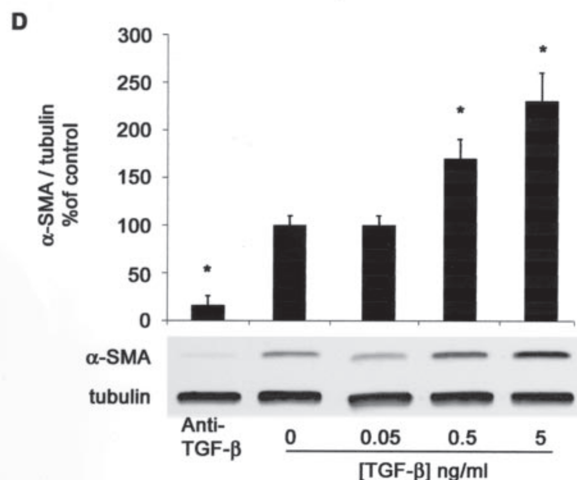

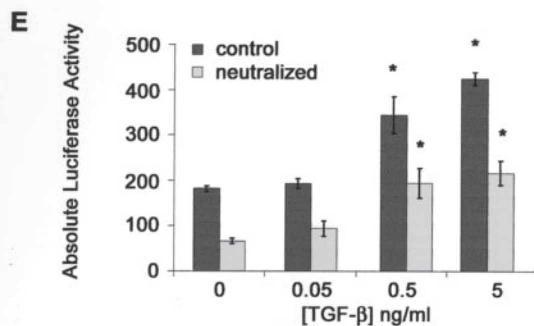

Figure 1. α-SMA expression in VICs. Immunofluorescent detection of α-SMA–containing stress fibers (green) at ×100 (A) and ×40 (B) magnification, and 4′,6-diamidino-2-phenylindole–stained nuclei (blue). Cells treated for 48 hours with neutralizing antibody exhibited reduced staining (C). Immunoblot analysis of TGF-β1 mediated changes in α-SMA protein levels (D) normalized to β-tubulin. E, Changes in α-SMA promoter activity 48 hours after TGF-β1 treatment. Neutralized cells (gray bars) were pretreated for 5 days with neutralizing antibody before TGF-β1 treatment.

sions.[46] After visualizing the impressive formation and alignment of α-SMA stress fibers mediated by TGF-β1, we predicted that activated valve myofibroblasts would exert increased tension on the ECM, specifically the fibronectin matrix. Indeed, TGF-β1 stimulation promoted considerable remodeling of the fibronectin matrix (Figure 2, fibronectin panels). This impressive reorganization suggests that TGF-β1 not only increases the contractile capacity of VICs but also enhances their ability to transmit force to the ECM, presumably through stronger focal adhesions. These results have noteworthy implications for valve remodeling because they illustrate the significant relationship between activated α-SMA–positive myofibroblasts and the extensive ECM disarray that is observed near myofibroblasts in diseased valves.

TGF-β and Mechanical Tension Synergistically Enhance VIC Force Generation

As Figure 2 illustrates, TGF-β1 appears to promote a contractile phenotype. Although vasoactivating agonists such as endothelin-1 and angiotensin II induce the contraction of intact valve tissue[47,48] and isolated VICs,[5] measurement of force generation by VICs after TGF-β treatment has not been reported. The contractile response of VICs to TGF-β1 was assayed using free-floating and stressed (mechanically loaded) collagen matrices.[49] Floating matrices mimic the normal connective tissue environment,[12,50] whereas stressed matrices induce the differentiation of myofibroblasts and more accurately mimic wound healing situations in which mechanical load is transferred from the matrix to migrating fibroblasts.[45] Without mechanical loading, TGF-β1 promoted the slow contraction of free-floating matrices by VICs, with a maximal response at 0.5 ng/mL (Figure 3A). Untreated matrices also contracted to some extent because of the reorganization of collagen fibers that occurs as cells migrate through the collagen matrix.[51] In contrast, stressed matrices displayed a rapid, dose-dependent TGF-β response after mechanical loading (Figure 3B). Concentrations of TGF-β1 >5 ng/mL did not further enhance matrix contraction (data

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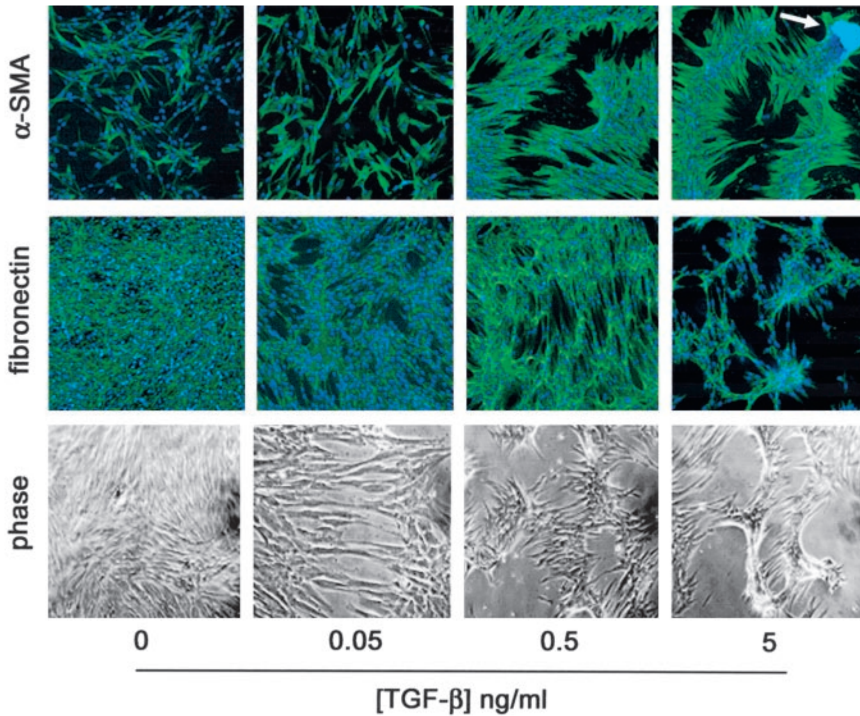

Figure 2. Cytoskeletal alterations during the early stages of TGF-β–mediated VIC aggregation. Cells cultured at high density were incubated in low-serum media (1% FBS) with or without TGF-β1 for 48 hours. Images are at ×10 magnification. Each series (α-SMA, fibronectin, and phase) was processed separately. Green, α-SMA or fibronectin fibrils; blue, 4′,6-diamidino-2-phenylindole–stained nuclei. Arrow denotes forming nodule.

not shown). More significantly, increased mechanical tension conferred a greater contractile response, with the reduction of matrix diameter occurring more rapidly, as determined by comparison of diameter changes between floating and stressed matrices (Figure 3C). Such comparison illustrates the cooperative effect TGF-β1 treatment and mechanical tension have on the VIC contractile phenotype. The enhanced contractile response of VICs also correlates strongly with a 230% increase in α-SMA levels observed after TGF-β1 treatment (Figure 1D). TGF-β1–induced contraction was dependent entirely on intact actin fibers; incubation of released matrices with cytochalasin D blocked TGF-β1–induced contraction (Figure 3D). Inhibition was reversible but delayed if cytochalasin D was added briefly for 30 minutes. Because the β1 and β3 isoforms of TGF-β regulate myofibroblast contractility,[52] contraction experiments were performed using TGF-β3 with similar results (data not shown). Hence, activation of valve myofibroblasts by TGF-β promotes a highly contractile phenotype that correlates strongly with increased α-SMA levels, enhanced stress fiber formation, and ECM disarray. This response is significantly enhanced by mechanical loading.

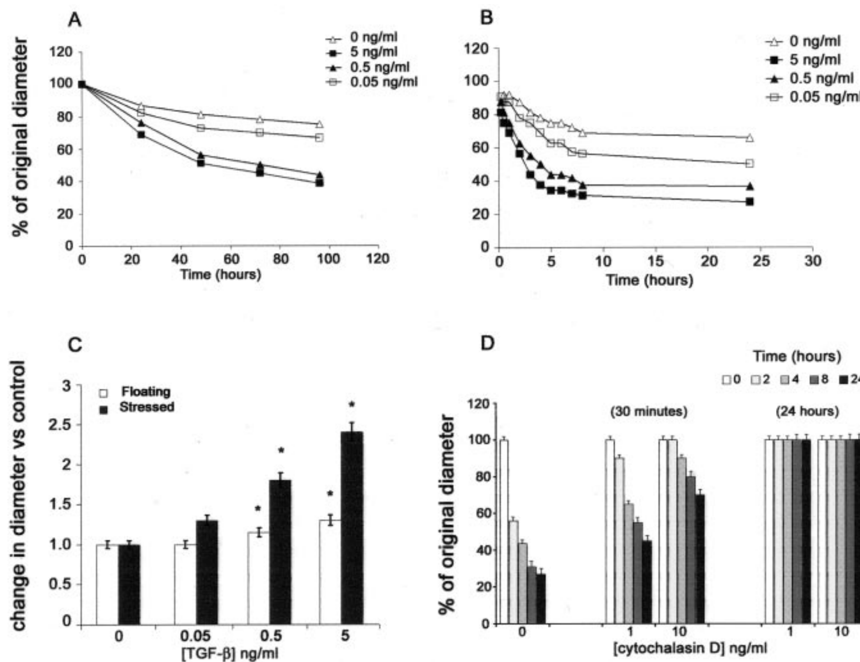

Figure 3. Collagen contraction assays. Changes in matrix size are plotted as percentage of the original diameter. A, Change in diameter vs time for floating matrices. B, Change in diameter of stressed matrices 24 hours after release. C, The extent of contraction is dramatically different between floating and stressed matrices and between TGF-β1–treated and TGF-β1–untreated matrices during the first 24 hours of measurement. D, Inhibition of contraction with cytochalasin D. TGF-β (5 ng/mL)–stimulated matrices were treated briefly (30 minutes) or continuously (24 hours).

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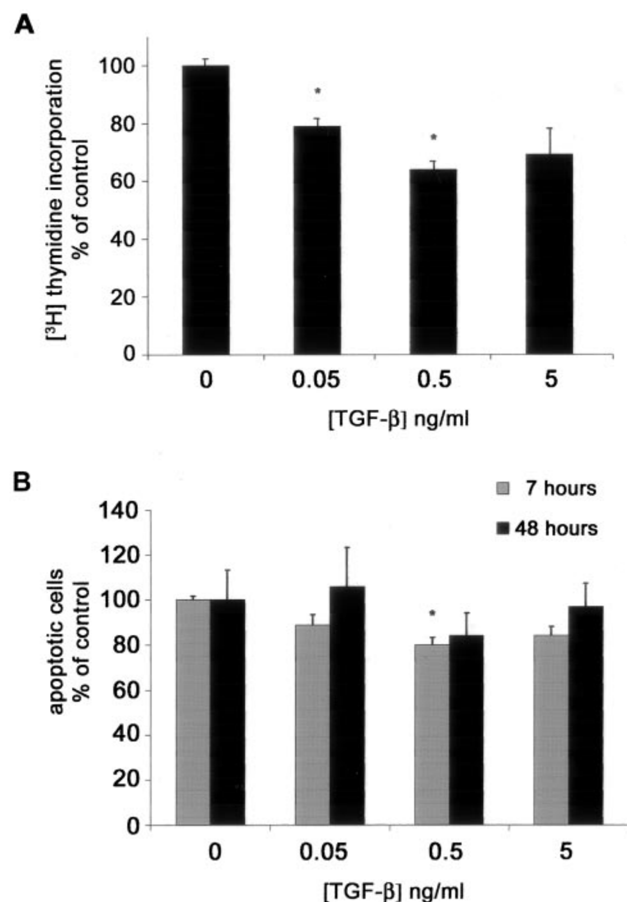

Figure 4. DNA synthesis and apoptosis. A, VIC DNA synthesis measured after 24 hours of [^{3}H]-thymidine incorporation. Data plotted as percentage change vs the untreated (0 ng/mL) control. B, Changes in apoptosis as measured by incorporation of a dye (APOPercentage Dye) into apoptotic cells. Results are plotted as percentage of dye incorporation normalized to the untreated control.

TGF-β1 Inhibits VIC Proliferation and Apoptosis

During the course of these experiments, it became apparent that TGF-β1 inhibited porcine aortic VIC proliferation, an observation that contradicted other reports.[9] To quantify our observation that TGF-β1 slows proliferation, the TGF-β effect on VIC DNA synthesis was measured by [^{3}H]-thymidine incorporation (Figure 4A). Under our culture conditions (ie, where cultures are <50% confluent), TGF-β1 clearly inhibited DNA synthesis by ≈40% at 0.5 ng/mL, with no further inhibition at 5 ng/mL of TGF-β. High concentrations of TGF-β1 (10 ng/mL) are capable of enhancing the rate of apoptosis of VICs under high-density conditions that stimulate VICs to migrate into large calcified nodules.[36] Given that our proliferation and α-SMA quantification experiments were performed with subconfluent cultures, we were compelled to assay changes in the rate of apoptosis using a system that identifies and quantifies cells that are committed to apoptosis. VIC apoptosis was not increased by 0.05 to 5 ng/mL of TGF-β1, either 7 or 48 hours after treatment (Figure 4B). Hence, when VICs are cultured at a density that does not result in nodule formation, TGF-β1 inhibits their proliferation without enhancing apoptosis.

Discussion

A role for myofibroblasts in cardiac valve remodeling, repair, and disease was originally proposed by Messier et al,[5] who recognized that the muscle-like properties of isolated VICs were similar to those of myofibroblasts described in granulation tissue and fibrocontractive diseases.[22] However, very little is known about what signals, chemical or mechanical, initiate valve myofibroblast differentiation. We set out to define the function of the potent myofibroblast activator TGF-β1 in this process. TGF-β1 is a member of the TGF-β cytokine superfamily that coordinates differentiation of mesenchymal stem cells during such distinct processes as organogenesis, bone and neuronal tissue formation, and myofibroblast activation.[54] We designed our experiments to specifically characterize the myofibroblast contractile phenotype, its induction by TGF-β, and the potential contribution of VICs to pathological changes in valve matrix independent of calcification, a process that is also induced by TGF-β.[34,36]

Here, we demonstrate that TGF-β1 is singularly capable of regulating valve myofibroblast activation and differentiation in vitro. We believe that the 2 phenotypes observed in untreated cultures (α-SMA–positive and α-SMA–negative) reflect local levels of active TGF-β secreted from VICs because we were able to manipulate the expression of α-SMA in all cells with either a TGF-β–neutralizing antibody or exogenous TGF-β1 (Figure 1B and 1C; Figure 2, α-SMA panels).

Myofibroblast activation is marked by increased cytokine and ECM synthesis, coupled with the development of a highly contractile cytoskeleton.[14,16] Others have shown recently that TGF-β1 stimulates ECM synthesis from sheep aortic VICs.[9] We have complemented these experiments by establishing that TGF-β1 signaling additionally confers enhanced contractile properties to VICs, promoting their differentiation into active valve myofibroblasts. TGF-β1 modulates VIC α-SMA expression in a dose-dependent manner by regulating α-SMA promoter activity. In turn, elevated α-SMA levels correlate strongly with enhanced force-generating capacities that are measurable with collagen matrix assays and dependent on an intact actin cytoskeleton.

Activation of myofibroblast contraction within valve tissue has implications for normal and pathological ECM remodeling. The observation that contractile myofibroblasts can drastically alter their surrounding fibronectin matrix in vitro is significant because it suggests that α-SMA–positive myofibroblasts may promote disease by exerting extensive remodeling forces on the valve matrix. That mechanical tension can exacerbate the effects of TGF-β1 on valve myofibroblast contractility is also intriguing. In vivo, cells are shielded from mechanical stress by the matrix they produce; however, when this protective shield is compromised by injury or disease, the stress load is then transferred to cells, which in turn respond by developing properties that will support the increased load.[12,45] Our results indicate that increased mechanical load alone can initiate the contractile phenotype of VICs. This, coupled with even small increases in local TGF-β1 concentrations, could contribute to the initiation of valve matrix disarray and degeneration in mechanically or biologically compromised valves.

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Myofibroblasts are extremely heterogeneous in nature and can express any combination of smooth and skeletal muscle–specific contractile and regulatory proteins[13,17] that may confer enhanced contractile properties. In VICs isolated from human valve cusps, mRNAs encoding the cardiac-specific isoforms of MyHC (cardiac α and β isoforms), troponin T, I, and C, and myosin light chain 2 have been detected,[41] but these results were not verified by protein analysis. Although others have established that sarcomeric proteins contribute to myofibroblast contraction,[20] our immunohistochemical analysis of porcine aortic VICs did not identify any of these striated muscle-specific proteins. Instead we detected myogenic factor 5 (myf-5), the expression of which varied from isolation to isolation (G.A.W., 2002, unpublished data). Myf-5 belongs to the family of skeletal muscle myogenic regulatory factors, the members of which can be expressed in kidney, liver, and lung myofibroblasts.[19,20] We conclude that the only muscle-specific protein expressed in VICs capable of conferring contractile properties is α-SMA. Notably, α-SMA protein is incorporated into stress fibers, the established contractile components of fibroblasts and myofibroblasts.[53]

Apoptosis is necessary for the elimination of activated myofibroblasts from tissue. Although TGF-β1 induces apoptosis in a wide variety of cell types, it can actually protect myofibroblasts from apoptotic stimuli. This protection is dependent on mechanical tension and an intact actin cytoskeleton,[55,56] attributes of VICs that are significantly enhanced by TGF-β1.[12] We observed that subconfluent cultures of VICs treated with TGF-β1 have fewer apoptotic cells than untreated cultures. However, this effect is reversed for confluent VICs that, in the presence of TGF-β1, rapidly form calcified nodules containing large numbers of apoptotic cells.[36] These contrasting observations indicate that TGF-β1 may impart distinct effects on the outcome of valve disease, depending on the extent of VIC proliferation and cell–cell contact within valve cusps.

Our experiments demonstrate the effect that small increases in TGF-β1 concentrations can have on valve myofibroblast differentiation and contractility. How might these in vitro experiments correlate with the activation of valve myofibroblasts in vivo? In vitro, TGF-β1 is increased by VIC treatment with serotonin[9,36] or angiotensin II,[57–59] agonists whose concentrations are locally increased in hearts from individuals with carcinoid syndrome, atherosclerotic plaques, or myocardial fibrosis.[24] TGF-β1 concentrations are also elevated at the site of valve lesions, similar to wounds in other tissues.[43] Finally, the inflammatory cells that infiltrate valve cusps during rheumatic fever and endocarditis are also a source of TGF-β1.[30] Our in vitro results suggest that VICs in vivo may be very sensitive to small changes in circulating TGF-β1, which will alter their biological activity. Our experimental conditions (0.05 to 5 ng/mL) correspond to molar concentrations of 10^{-10} mol/L to 10^{-12} mol/L, respectively, indicating that picomolar changes in circulating TGF-β1 could have profound effects on the biological activity of VICs in vivo. With increased interest in engineering of valve tissue equivalents, the ability to control activation and deactivation of VICs on synthetic 3D culture environments has become increasingly important for regeneration of functional tissue.[29] Our understanding of the critical role that TGF-β1 plays in this process will contribute to future strategies.

By further characterizing the response of VICs to TGF-β1 in vitro, we have been able to establish correlation between the appearance of α-SMA myofibroblasts and valve matrix disorganization. Given the high levels of mechanical stress to which valves are chronically exposed, it is evident that very small changes to VIC–matrix dynamics induced by increases in TGF-β signaling may accumulate quickly into large, pathological changes in valve architecture and ultimately result in valve disease.

Acknowledgments

This study was supported by National Institutes of Health grant GM-29090 and the Packard Foundation. We thank Jamie Sheren and Charles Nuttelman for their contributions.

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Supplemental Data

Antibodies used for immunohistochemistry and immunoblot

The antibodies listed are known to react with porcine proteins. Monoclonal anti- α-smooth muscle actin (anti-alpha actin, clone 1A4, ICN Biomedical); monoclonal anti-β1-tubulin (Sigma Chemical, clone SAP.4G5), monoclonal fibronectin (Sigma Chemical, clone FN-3E2); polyclonal anti-desmin (Biomeda, cat # V2022); monoclonal anti-sarcomeric myosin heavy chain, (clone F59, gift of Dr. F. Stockdale, Stanford University); monoclonal anti-vimentin (Sigma Chemical, cloneV9); smooth muscle myosin heavy chain (Chemicon clone ID8,); sarcomeric troponin T (Sigma Chemical, clone, JLT-12); rabbit polyclonal Myf-5 (Santa Cruz Biotechnology, SC-302); mouse monoclonal MyoD (Novagen, clone 5.8A); and monoclonal myogenin antibody (Developmental Hybridoma Bank at the University of Iowa, clone F5D).

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