Transforming Growth Factor-β Function Blocking Prevents Myocardial Fibrosis and Diastolic Dysfunction in Pressure-Overloaded Rats

Fumitaka Kuwahara, MD; Hisashi Kai, MD, PhD; Keisuke Tokuda, MD; Mamiko Kai, PhD; Akira Takeshita, MD, PhD; Kensuke Egashira, MD, PhD; Tsutomu Imaizumi, MD, PhD

Background—Excessive myocardial fibrosis impairs cardiac function in hypertensive hearts. Roles of transforming growth factor (TGF)-β in myocardial remodeling and cardiac dysfunction were examined in pressure-overloaded rats.

Methods and Results—Pressure overload was induced by a suprarenal aortic constriction in Wistar rats. Fibroblast activation (proliferation and phenotype transition to myofibroblasts) was observed after day 3 and peaked at days 3 to 7. Thereafter, myocyte hypertrophy and myocardial fibrosis developed by day 28. At day 28, echocardiography showed normal left ventricular fractional shortening, but the decreased ratio of early to late filling velocity of the transmitral Doppler velocity and hemodynamic measurement revealed left ventricular end-diastolic pressure elevation, indicating normal systolic but abnormal diastolic function. Myocardial TGF-β mRNA expression was induced after day 3, peaked at day 7, and remained modestly increased at day 28. An anti–TGF-β neutralizing antibody, which was administered intraperitoneally daily from 1 day before operation, inhibited fibroblast activation and subsequently prevented collagen mRNA induction and myocardial fibrosis, but not myocyte hypertrophy. Neutralizing antibody reversed diastolic dysfunction without affecting blood pressure and systolic function.

Conclusions—TGF-β plays a causal role in myocardial fibrosis and diastolic dysfunction through fibroblast activation in pressure-overloaded hearts. Our findings may provide an insight into a new therapeutic strategy to prevent myocardial fibrosis and diastolic dysfunction in pressure-overloaded hearts. (Circulation. 2002;106:130-135.)

Key Words: growth substances • fibrosis • hypertension • diastole

Diastolic dysfunction is typically seen in patients with hypertensive heart disease or hypertrophic cardiomyopathy and has a particularly high prevalence in elderly patients.1 Although the prognosis of diastolic heart failure is usually better than that of heart failure with systolic dysfunction, morbidity can be substantial.1 Disproportional accumulation of fibrous tissue is a major determinant of impaired stiffness and pumping capacity in hypertrophied hearts, and its excessive accumulation accounts for a spectrum of ventricular dysfunction that first appears during diastole and subsequently involves systole.2 Appropriate therapy of diastolic dysfunction is desirable, but no specific therapy is available to improve left ventricular (LV) diastolic function at present.

Transforming growth factor (TGF)-β is a locally generated cytokine that has been implicated as a major stimulator of tissue fibroinflammatory changes.3 TGF-β has a major influence on fibroblast proliferation and extracellular matrix production, particularly of collagen and fibronectin, while reducing degradation of these components.3 TGF-β also modulates the phenotypic conversion of fibroblasts to myofibroblasts that express α-smooth muscle actin (SMA).4 Myofibroblasts play a role in a wide range of pathological conditions associated with fibrosis and organ remodeling by actively producing extracellular matrix components as well as profibrotic mediators, including TGF-β.5 Recent studies in humans and experimental models have shown increased myocardial TGF-β expression during cardiac hypertrophy and fibrosis.6–8 Therefore, TGF-β is thought to exert a role in myocardial fibrosis in pressure-overloaded (PO) hearts. A causal relation between TGF-β and myocardial remodeling, however, remains undetermined in PO hearts.

Anti–TGF-β neutralizing antibodies (NABs) are very effective tools to investigate the roles of TGF-β in the pathogenesis of various experimental disease models by inhibiting TGF-β activity in the kidney, skin, and arterial wall.9–11 It has been shown that an anti–pan–TGF-β monoclonal NAB effectively prevents the expression of extracellular matrix proteins.
and subsequent myocardial fibrosis in rats with long-term blockade of nitric oxide synthesis.\textsuperscript{12} Therefore, using the NAb, we investigated the roles of TGF-β in myocardial remodeling in PO rats with a suprarenal abdominal aortic constriction (AC). Also, we examined whether prevention of myocardial remodeling by blocking TGF-β function would improve cardiac dysfunction in PO rats.

Methods
All procedures were in accordance with institutional guidelines of animal care and treatment. After male Wistar rats (8 weeks old) had been anesthetized with pentobarbital (50 mg/kg IP), AC or the sham operation (sham) was established.\textsuperscript{13} Blood pressure was measured in the unrestricted, conscious state through a heparinized indwelling polyethylene catheter that was introduced into the left carotid artery 1 day before measurement.\textsuperscript{13} Unless otherwise indicated, 6 rats were studied in each group for each time point.

Protocol 1
Tissue Preparation and Morphometry
Rats were killed with an overdose of pentobarbital. Blood was drawn from the right ventricle for plasma renin activity measurement,\textsuperscript{14} and the rats were perfusion-fixed with 4% paraformaldehyde in Hanks’ solution at 100 mm Hg. The LV was carefully isolated, weighed, embedded in paraffin, and cut into 5-μm cross sections. To evaluate myocyte hypertrophy and myocardial fibrosis, 3 independent hematoxylin-eosin–stained and 3 Mallory-Azan–stained sections from each rat were examined and analyzed with a digital image analyzer. The shortest transverse myocyte diameter was measured in 50 nucleated transverse sections of the myocytes in each tissue section.\textsuperscript{15} The percent area of myocardial fibrosis (% myocardial fibrosis) was calculated as previously described.\textsuperscript{16}

Immunohistostaining and In Situ BrdU Labeling
In situ bromodeoxyuridine (BrdU) labeling was performed to identify the proliferating cells.\textsuperscript{17} BrdU/‘vimentin’ and SMA/‘vimentin’-spindle-shaped cells were defined as proliferating fibroblasts and myofibroblasts, respectively, by use of double immunostaining with a double-immunostain kit (DAKO).\textsuperscript{17} The labeled cells were counted at ×200 magnification in 4 independent entire cross sections of each animal. For anti–TGF-β immunohistostaining, perfusion-fixation was performed with methacarn solution,\textsuperscript{18} and the sections were subjected to immunohistostaining with an LSAB2 kit (Dako).\textsuperscript{19}

Quantitative Real-Time RT-PCR
Quantitative analysis of the target mRNA expression was performed with real-time TaqMan reverse transcription–polymerase chain reaction (RT-PCR) by the relative standard curve method. Total RNA was extracted from unfixed hearts with TRIzol (GIBCO BRL) followed by RNase-free DNase I (Boehringer). Aliquots (25 ng) of the total RNA were reverse-transcribed and amplified in triplicate with the TaqMan rodent GAPDH control reagents (PE Biosystems), according to the manufacturer’s instructions. The expression level of the target gene was determined from the relative standard curves constructed with serial dilutions of the control total RNA (PE Biosystems) according to the manufacturer’s instructions. The expression level of the target gene was normalized by the GAPDH level in each sample.

Echocardiographic Studies
Transthoracic echocardiographic studies were performed at day 28 with a commercially available echocardiographic machine equipped with a 10-MHz transducer (Aloka). Rats were slightly anesthetized with ketamine (50 mg/kg IP) and xylazine (10 mg/kg IP). M-mode tracings were recorded at the level of the papillary muscles, and LV mass and fractional shortening were calculated.\textsuperscript{20} The ratio of early to late filling velocity (E/A) of transmitral pulse-wave Doppler spectra was measured as described previously.\textsuperscript{21}

Statistical Analysis
Quantitative analysis was performed by a single observer in a blinded manner. One-way ANOVA followed by Scheffe’s F test was performed for the statistical comparisons. A value of $P<0.05$ was considered significant.

Results
Protocol 1
AC induced significant and sustained elevation of mean arterial pressure (MAP) within 1 day, whereas MAP did not change in sham rats (Figure 1A). In AC rats, the LV weight/body weight ratio (LVW/BW), a parameter of LV hypertrophy, increased progressively after day 7, whereas sham rats showed no significant LVW/BW changes (Figure 1B). AC did not affect plasma renin activity levels during the observation period (Figure 1C).
Myocardial Remodeling

In AC rats, the transverse diameter of cardiac myocytes was similar to that in sham rats by day 3 and increased progressively after day 7, reaching 164% of that of sham rats at day 28 (Figure 1D). Sham rats did not show significant changes in the myocyte diameter.

In sham rats, the Azan-stained fibrotic area was sparse in the interstitial space, and the number of vimentin+ fibroblasts was small. Almost no fibroblasts were labeled with BrdU or SMA. In AC rats, progressive interstitial changes were observed, characterized by increased cellularity and interstitial fibrosis (Figure 2A). At day 3, a robust increase in BrdU+ fibroblasts was observed in the perivascular interstitium. At day 7, BrdU+ fibroblasts were observed in not only the perivascular but also the intermuscular space. Also, double immunohistostaining with vimentin and SMA showed colocalization of myofibroblasts with proliferating fibroblasts (data not shown). The increase in myofibroblasts may have resulted from enhanced phenotypic transition of fibroblasts to myofibroblasts and in part from the absolute increase in the myofibroblasts with proliferating fibroblasts (Figure 2B).

TGF-β Expression

Real-time RT-PCR analysis demonstrated that in AC rats, myocardial TGF-β mRNA expression was significantly upregulated after day 3, peaked at day 7, and remained moderately increased at day 28 (Figure 3A). TGF-β expression remained unchanged in sham rats. Furthermore, at day 7, AC rats showed immunoreactive TGF-β in the interstitium in a distribution similar to that of BrdU+ fibroblasts and myofibroblasts (Figure 3B).

Protocol 2

To investigate the effects of TGF-β function blocking, NAb or control IgG was administered daily from 1 day before operation. The AC-induced MAP elevation was not different between AC+IgG and AC+NAb rats (191±7 and 186±8 mm Hg, respectively, at day 28). AC+NAb rats showed smaller LVW/BW than AC+IgG rats, although the change was not statistically significant (2.7±0.2 and 3.0±0.2, respectively, at day 28). Control IgG did not affect these parameters in AC and sham rats. Apparently, no adverse effects were observed in rats treated with NAb or IgG.

TGF-β Neutralization and Myocardial Remodeling

Myocyte diameter and % myocardial fibrosis did not differ between AC and AC+IgG rats at day 28. At day 28, NAb remarkably decreased the AC-induced myocardial fibrosis and induction of type I and III collagens, while not affecting the AC-induced myocyte hypertrophy (Figures 4 and 5). Furthermore, NAb remarkably decreased the AC-induced increases in BrdU+ fibroblasts and myofibroblasts at day 3 (Figure 6). These parameters were not significantly different between AC and AC+IgG rats. Microscopic examination revealed no other apparent abnormality of the myocardial architecture in AC+NAb rats. Moreover, neither macroscopic nor microscopic abnormalities were found in the hearts of sham+IgG or sham+NAb rats.

TGF-β Neutralization and Cardiac Function

Echocardiographic and hemodynamic studies were performed in sham, AC+NAb, and AC+IgG rats at day 28 (Figure 7). Heart rate and MAP were not significantly different among the 3 groups. In AC+IgG rats, M-mode echocardiography demonstrated concentric LV hypertrophy, whereas fractional shortening was similar to that in sham rats.
In AC/H11001 IgG rats, transmitral Doppler velocity showed decreased early and increased late filling velocities, resulting in E/A reduction, and LVEDP increased significantly compared with sham rats. NAb reversed E/A and LVEDP to levels not significantly different from those of sham rats, without affecting fractional shortening. Echocardiographic LV mass of AC/H11001 NAb rats was smaller than that of AC/H11001 IgG rats, but not significantly so.

**Discussion**

The present study showed that fibroblast activation (proliferation and phenotypic transition to myofibroblasts) and TGF-β expression were induced in PO rat hearts after day 3, with a peak at days 3 to 7, and thereafter myocardial fibrosis progressed, resulting in diastolic, but not systolic, dysfunction at day 28. Furthermore, TGF-β function blocking not only inhibited fibroblast activation and myocardial fibrosis but also prevented diastolic dysfunction, while not affecting MAP, myocyte hypertrophy, and systolic function.
Excessive myocardial fibrosis has been implicated in progression of cardiac dysfunction, especially diastolic dysfunction, in hypertensive hearts.\(^1,2,20\) Thus, we used a PO model produced by constricting the suprarenal abdominal aorta of Wistar rats. This model is characterized by a rapid progression of marked myocardial fibrosis associated with diastolic dysfunction. Effects of circulating renin-angiotensin system activation on cardiac remodeling can be eliminated in this model, because plasma renin activity was unchanged.

The initial event of myocardial remodeling in response to PO was manifested by fibroblast activation and TGF-\(\beta\) induction (Figures 2 and 3). Also, immunoreactive TGF-\(\beta\) was apparently distributed in the interstitium similarly to proliferating fibroblasts and myofibroblasts. It was noteworthy that fibroblast activation and TGF-\(\beta\) induction occurred before significant myocardial fibrosis developed, and furthermore, increased TGF-\(\beta\) expression was sustained during the progression of myocardial fibrosis (Figure 3A). These findings are consistent with earlier observations that TGF-\(\beta\) induction preceded the induction of extracellular matrix components, including collagen and fibronectin, in PO hearts.\(^6\) In addition, TGF-\(\beta\) self-amplifies TGF-\(\beta\) expression in fibroblasts and myofibroblasts.\(^5\) Therefore, our study suggests that TGF-\(\beta\) plays a crucial role as a mediator of myocardial fibrosis in PO hearts. As expected, TGF-\(\beta\) function blocking with NAb dramatically prevented not only fibroblast activation but also collagen mRNA induction and myocardial fibrosis (Figures 4 to 6), clearly underlining the causal relation of TGF-\(\beta\) to myocardial fibrosis through fibroblast activation in PO hearts. Because NAb had no effects on MAP elevation in AC rats, it is suggested that the inhibitory effects of NAb on myocardial fibrosis are not related to hemodynamic changes. It is unlikely that TGF-\(\beta\) has the major impact on compensatory hypertrophy of the adult myocyte in response to pressure overload in this model, although TGF-\(\beta\) was reported to induce growth of cultured neonatal cardiac myocytes.\(^21\) The effects of NAb on LV hypertrophy may be attributable to the reduction in myocardial fibrosis, because NAb did not affect myocyte hypertrophy and because the decrease in echocardiographic LV mass (15% reduction; Figure 7B) seems similar to that in % myocardial fibrosis (18% reduction; Figure 5A).

Myocardial fibrosis and myocyte hypertrophy have been implicated in increased myocardial stiffness, resulting in diastolic dysfunction, in hypertensive hearts.\(^2,20\) In the present study, PO rats showed a decreased E/A of transmural Doppler velocity accompanied by elevated LVEDP (Figure 7). The decreased E/A indicates impairment of early diastolic LV filling with a compensatory late diastolic filling caused by enhanced atrial contraction.\(^22\) Furthermore, LVEDP elevation, which is the end result of diastolic dysfunction, is caused by a decrease in the effective operative LV compliance attributable to wall thickening and/or increased myocardial stiffness resulting from myocardial fibrosis.\(^19,22\) The present study clearly demonstrated that TGF-\(\beta\) function blocking ameliorated diastolic dysfunction in PO hearts without affecting MAP and myocyte hypertrophy. Taken together, these results suggest that the TGF-\(\beta\)-mediated myocardial fibrosis is the major determinant of impaired diastolic function, probably through increased myocardial stiffness in this model. NAb did not, however, completely reverse diastolic dysfunction. This may be related to the fact that significant LV and myocyte hypertrophy remained in AC+NAb rats, because these factors increase passive stiffness and impair LV relaxation.\(^19,22\)

It is noteworthy that NAb preserved the cardiac morphology and systolic function and induced no apparent microscopic changes in the myocardial architecture in sham rats. These observations are in line with earlier reports that NAb against TGF-\(\beta\) prevent excess amounts of extracellular matrix deposition but do not interfere with normal healing and function of the tissue in various models of tissue fibrosis.\(^9,10\) Therefore, NAb may have inhibitory effects on the sequel of the excessive fibrosis in response to pressure overload but would have minimum effects on physiological function of extracellular matrix proteins, such as the maintenance of normal myocardial architecture and the transmission of force generated by myocytes to the ventricular chamber.\(^2\)

The precise biochemical triggers responsible for TGF-\(\beta\) induction are currently unknown in PO hearts. Recent studies hypothesized that hemodynamic stress triggers a proinflammatory process around the intracardiac vasculature and activates endothelial cells, vascular smooth muscle cells, and cardiac myocytes as well as perivascular infiltrating inflammatory cells. In turn, these cells are considered to produce proinflammatory and fibrogenic mediators, leading to fibroblast activation and myocardial fibrosis.\(^23\) Our observations that the AC-induced fibroblast proliferation and myocardial fibrosis were initiated in the perivascular space may support this hypothesis. Mechanical stress and locally generated cytokines or growth factors, including angiotensin II, endothelin, and tumor necrosis factor-\(\alpha\), would be expected to be candidates for TGF-\(\beta\) induction and activation.\(^3,7,24\) Because TGF-\(\beta\) function blocking dramatically prevented myocardial fibrosis, it is likely that TGF-\(\beta\) is located downstream of these various fibrogenic pathways activated in response to PO. Our observation that there was a time lag between the initiation of PO and the induction of TGF-\(\beta\) mRNA expression may indirectly support this hypothesis. A cellular source of TGF-\(\beta\) in hypertrophied hearts, however, currently remains controversial.\(^3,7,24\) Accordingly, future studies should focus on the cellular and molecular mechanisms of TGF-\(\beta\) induction in PO hearts.

**Limitations**

Various growth factors and cytokines exist in the heart and are supposed to be induced during cardiac hypertrophy.\(^2,23\) Although the present study clearly showed an important role of TGF-\(\beta\), it is possible that other factors are involved in the mechanisms of myocardial fibrosis and diastolic dysfunction. Recently, Nakajima et al\(^25\) reported that transgenic mice overexpressing a constitutively active TGF-\(\beta\) mutant show atrial but not ventricular fibrosis, although active TGF-\(\beta\) levels are similar in the atria and ventricles. Currently, the reason for the conflicting results regarding the role of TGF-\(\beta\) in ventricular fibrosis is unknown, although the discrepancy may be caused by differences between the animal models used in our and their studies. Effects of active TGF-\(\beta_1\)
overexpression on AC-induced myocardial fibrosis should be addressed in future studies.

In conclusion, TGF-β is suggested to play a key role in myocardial fibrosis in PO hearts through fibroblast activation. Also, inhibition of TGF-β-mediated myocardial fibrosis prevents progression of diastolic dysfunction in this model. The present study should provide insight into new therapeutic strategies to prevent cardiac fibrosis and to preserve diastolic function, which may prevent further progression to diastolic and subsequent systolic heart failure and death, in hypertensive hearts.

Acknowledgments

This study was supported in part by a grant from the Science Frontier Research Promotion Centers and Grants-in-Aid for Scientific Research (Drs Kuwahara, H. Kai, and M. K.; by a Ministry of Education, Science, Sports, and Culture, Japan; by a Kimura Memorial Foundation grant (Dr H. Kai); and by a Mochida Memorial Heart Foundation research grant (Dr Kuwahara); by an Ishibashi Education, Science, Sports, and Culture, Japan; by a Kimura Memorial Heart Foundation research grant (Dr Kuwahara); by an Ishibashi Education, Science, Sports, and Culture, Japan; by a Kimura Memorial Heart Foundation research grant (Dr Kuwahara); by an Ishibashi Research Grant (Dr Kai); and by a Mochida Memorial Heart Foundation research grant (Dr H. Kai). We thank Kaoru Moriyama and Yayoi Yoshida for technical assistance.

References

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_Circulation_. 2002;106:130-135; originally published online June 10, 2002;
doi: 10.1161/01.CIR.0000020689.12472.E0

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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