Hypertension
Journal of the American Heart Association

Early Induction of Transforming Growth Factor-β via Angiotensin II Type 1 Receptors Contributes to Cardiac Fibrosis Induced by Long-term Blockade of Nitric Oxide Synthesis in Rats

Hideharu Tomita, Kensuke Egashira, Yuichi Ohara, Masao Takemoto, Masamichi Koyanagi, Makoto Katoh, Hiroaki Yamamoto, Kiyoshi Tamaki, Hiroaki Shimokawa and Akira Takeshita

Hypertension. 1998;32:273-279
doi: 10.1161/01.HYP.32.2.273
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0194-911X. Online ISSN: 1524-4563

Early Induction of Transforming Growth Factor-β via Angiotensin II Type 1 Receptors Contributes to Cardiac Fibrosis Induced by Long-term Blockade of Nitric Oxide Synthesis in Rats

Hideharu Tomita, Kensuke Egashira, Yuichi Ohara, Masao Takemoto, Masamichi Koyanagi, Makoto Katoh, Hiroaki Yamamoto, Kiyoshi Tamaki, Hiroaki Shimokawa, Akira Takeshita

Abstract—We previously reported that the chronic inhibition of nitric oxide (NO) synthesis increases cardiac tissue angiotensin-converting enzyme expression and causes cardiac fibrosis in rats. However, the mechanisms are not known. Transforming growth factor-β (TGF-β) is a key molecule that is responsible for tissue fibrosis. The present study investigated the role of TGF-β in the pathogenesis of cardiac fibrosis. The development of cardiac fibrosis by oral administration of the NO synthesis inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) to normal rats was preceded by increases in mRNA levels of cardiac TGF-β_1 and extracellular matrix (ECM) proteins. TGF-β immunoreactivity was increased in the areas of fibrosis. Treatment with a specific angiotensin II type 1 receptor antagonist, but not with hydralazine, completely prevented the L-NAME–induced increases in the gene expression of TGF-β_1 and ECM proteins and also prevented cardiac fibrosis. Intraperitoneal injection of neutralizing antibody against TGF-β did not affect the L-NAME–induced increase in TGF-β_1 mRNA levels but prevented an increase in the mRNA levels of ECM protein. These results suggest that the early induction of TGF-β_1 via the angiotensin II type 1 receptor plays a major role in the development of cardiac fibrosis in this model. **(*Hypertension*. 1998;32:273-279.)**

Key Words: endothelium-derived relaxing factor ■ remodeling ■ growth substances ■ collagen ■ angiotensin II

Nitric oxide derived from the endothelium is a multifunctional substance that regulates vascular tone, platelet aggregation, thrombus formation, leukocyte adhesion, and vascular proliferation.[1–5] Evidence suggests that cardiovascular disorders such as hypertension, hypercholesterolemia, atherosclerosis, and aging are associated with endothelial dysfunction that leads to the abnormal production/release of NO.[1–9] Thus, defective NO synthesis may contribute to the structural changes in the coronary vasculature and myocardium in certain pathological conditions in vivo.

We recently showed that long-term blockade of NO synthesis with chronic oral administration of L-NAME increases cardiac tissue ACE activity as well as AT_1 receptor and causes cardiac remodeling (perivascular fibrosis, myocardial fibrosis, and vascular medial thickening).[10–13] We[12] and other investigators[14] have also reported that treatment with ACE inhibitors prevents such vascular and myocardial structural changes in this model, suggesting that overexpression of ACE and AT_1 receptor is important in the pathogenesis of cardiac remodeling. However, the molecular mechanisms of the cardiac fibrosis in this model are unknown.

Cardiac fibroinflammatory changes are thought to be an important pathological process in several models of hypertension.[15–18] Recent evidence suggests that TGF-β is a key factor responsible for tissue fibroinflammatory changes[19,20] because in vivo gene transfer of TGF-β induces fibrosis in rat lung[19] and kidney.[20] TGF-β stimulates the synthesis of ECM components such as collagen, fibronectin, and proteoglycan and inhibits ECM degradation.[21–23] The net effect of TGF-β thus leads to fibrosis. Increased expression of TGF-β has been shown in human and experimental cardiac hypertrophy/fibrosis.[24–26] Angiotensin II has been shown to induce ECM synthesis through the increased expression of TGF-β in cultured cardiac fibroblasts, vascular smooth muscle cells, and renal mesangial cells.[27–29] Thus, it is possible that angiotensin II–induced TGF-β expression may contribute to the cardiac fibrosis observed in the animal model with long-term inhibition of NO synthesis.

TGF-β is usually secreted as a biologically inactive or latent form.[30] Latent TGF-β is composed of mature TGF-β, which is biologically active, the latency-associated peptide, which is sufficient for the latency of TGF-β, and latent TGF-β binding protein. The latency-associated peptide is cleaved from TGF-β by proteolytic processing but remains associated TGF-β. The dissociation of the peptide renders TGF-β biologically active. Acidification, heating, and pro-

Received December 15, 1997; first decision January 8, 1998; revision accepted March 5, 1998.

From the Research Institute of Angiocardiology and the Second Department of Internal Medicine (K.T.), Kyushu University Faculty of Medicine, Fukuoka, Japan.

Correspondence to Kensuke Egashira, MD, PhD, Research Institute of Angiocardiology and Cardiovascular Clinic, Kyushu University School of Medicine, 3–1-1, Maidashi, Higashi-ku, Fukuoka 812–82, Japan. E-mail egashira@cardiol.med.kyushu-u.ac.jp

Selected Abbreviations and Acronyms

ACE = angiotensin-converting enzyme
AT_1 = angiotensin II type 1 receptor
ECM = extracellular matrix
L-NAME = N^{ω}-nitro-L-arginine methyl ester
NO = nitric oxide
TGF-β = transforming growth factor-β

tease treatment lead to activation of latent TGF-β in vitro, although it is still unknown how the latent complex is activated in vivo.

The purpose of this study was to investigate the mechanisms of cardiac fibrosis induced in rats by long-term administration of NO synthesis inhibitor. Our specific goals were to examine whether (1) cardiac fibrosis is associated with induction of mRNA for TGF-β_1 and ECM proteins; (2) the selective antagonist for AT_1 receptors prevents cardiac fibrosis and induction of mRNA for TGF-β_1 and ECM proteins; and (3) treatment with neutralizing antibody against TGF-β modulates the mRNA levels for TGF-β_1 and ECM proteins.

Methods

The present experiments were reviewed and approved by the Committee on Ethics in Animal Experiments, Faculty of Medicine, Kyushu University, and conducted according to the guidelines for animal experimentation of the Faculty of Medicine, Kyushu University, and Law (No. 105) and Notification (No. 6) of the Japanese Government.

Drugs

In this study, we used L-NAME (Sigma Chemical Co), L-arginine (Sigma), hydralazine (Ciba-Geigy Pharmaceutical Co), and CS-866, a specific AT_1 receptor antagonist (Sankyo Pharmaceutical Co) (Figure 1).[31]

Experimental Protocol 1

This study measured cardiac mRNA levels of TGF-β_1, fibronectin, and type 1 collagen.

Experimental Groups

Male Wistar-Kyoto rats were obtained from an established colony at the Animal Research Institution of Kyushu University Faculty of Medicine. Five groups of rats were studied. The control group received untreated chow and drinking water. The second group (L) received L-NAME in its drinking water (1 mg/mL). At this concentration, the daily intake of L-NAME for the latter group was 100 mg/kg per day. The third group (L+Hyd) received L-NAME and hydralazine (0.12 mg/mL) in its drinking water. The fourth group (L+CS-866) received L-NAME in its drinking water and CS-866 (75 μg/g) in its chow. The fifth group (L+L-arg) received L-NAME and L-arginine (70 mg/mL) in its drinking water. The doses of CS-866 and L-arginine were determined empirically, and we found in preliminary studies that oral administration of either CS-866 at 75 μg/g or L-arginine at 70 mg/mL alone for 7 days did not significantly affect systolic arterial blood pressure or TGF-β_1 mRNA levels. All rats were single-housed in a special pyrogen-free facility. We verified that rats drank 30 to 40 mL of water and ate 20 g of chow per day regardless of the treatment and also confirmed that their drinking and eating patterns were unaffected by any treatment protocol.

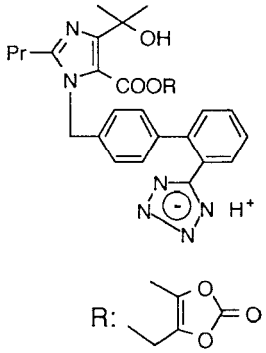

Figure 1. Chemical structure of the specific AT_1 receptor antagonist CS-866: (5-methyl-2-oxo-1,3-dioxolen-4-yl)methoxy-4-(1-hydroxy-1-methylethyl)-2-propyl-1-{4-[2-(tetrazol-5-yl)-phenyl]phenyl}methylimidazol-5-carboxylate.

Before treatment and 3 days, 1 week, and 8 weeks during L-NAME treatment, systolic blood pressure (by the tail-cuff method), heart rate, and body weight were measured. On the third day, first week, and eighth week of treatment, cardiac mRNA levels were measured in all 5 groups (all groups, n=7 to 8 in each time point). The rats were anesthetized with intraperitoneal pentobarbital. A blood sample was taken from the femoral artery, collected into a syringe, centrifuged, and then frozen at −20°C. Then the chest was opened, the heart was rapidly removed, and the atria, the great vessels, and right ventricle were trimmed away. The left ventricle was cut, put into liquid nitrogen, and stored at −80°C.

RNA Preparation and Northern Blot Analysis

Total RNA was isolated from the rat heart using ISOGEN (Nippon Gene, Tokyo) based on the acid guanidium thiocyanate–phenol–chloroform extraction method. Poly(A)+ RNA purification was carried out with an oligo(dT)–cellulose column (Takara Shuzo Co). Aliquots (5 μg) of poly(A)+RNA were denatured and size-fractionated by electrophoresis on a 1.0% agarose-formaldehyde gel, transferred to nylon membranes (Hybond N^+, Amersham), and immobilized by UV irradiation.

The membranes were prehybridized in a solution containing 50% formamide, 5× SSPE (0.9 mol/L sodium chloride, 0.05 mol/L sodium phosphate, and 0.005 mol/L EDTA), 5× Denhardt's solution (0.2% polyvinylpyrrolidone, 0.2% BSA, and 0.2% Ficoll), 0.5% SDS, and 100 μg/mL sonicated salmon sperm DNA at 42°C for at least 2 hours. The cDNA probes were labeled with [^{32}P]dCTP (3000 Ci/mmol, NEN) using a BcaBEST labeling kit (Takara Shuzo). The membranes were then hybridized with specific DNA probes in the same solution at 42°C overnight. The membranes were washed twice in 2× SSC at 42°C, once in 2× SSPE containing 0.1% SDS at 55°C, and once in 1× SSPE containing 0.1% SDS at 55°C for 15 minutes. Autoradiography was performed by standard methods. Relative amounts of mRNA were normalized against mouse GAPDH mRNA.

cDNA Probes

The cDNA probes used were as follows: a 1.23-kb *Eco*RI-*Eco*RI fragment of rat TGF-β_1 cDNA,[32] a 1.8-kb *Eco*RI-*Eco*RI fragment of human type I collagen cDNA (American Type Culture Collection), a 2.5-kb *Bam*HI-*Hin*dIII fragment of human fibronectin cDNA (provided by Dr F.E. Baralle), and a 1.3-kb *Pst*I-*Pst*I fragment of mouse GAPDH (American Type Culture Collection).

Experimental Protocol 2

In this protocol, histopathology and morphometry were performed at the eighth week of treatment in the 5 groups described in protocol 1 (all groups, n=8). Immunohistochemistry was performed in control (n=5) and the L groups (n=5) at the eighth week of treatment.

Fixation and Staining Procedure

Animals were anesthetized by intraperitoneal pentobarbital. Then the abdomen was opened and the abdominal aorta was cannulated. Next, the chest was opened and the right atrium was cut. The heart was perfused via the aorta with oxygenated Krebs-Henseleit solution at a pressure of 90 mm Hg, and the coronary vasculature was fixed with 6% formaldehyde or with methacarn solution.[33] After fixation, the left ventricle was separated from the atria, the great vessels, and the right ventricle. The left ventricle was cut into 5 pieces perpendicular to the long axis. Tissues were dehydrated, embedded in paraffin, cut

into slices 5 μm thick, and mounted on slides. Sections of formaldehyde-fixed tissues were stained with Masson's trichrome staining solutions for histopathological morphometry. Sections of methacarn-fixed tissues were used for immunohistochemistry.

Morphometry

The following morphometry was performed by a single observer who was blinded to all treatment protocols. Myocardial interstitial fibrosis was determined by quantitative morphometry as described.[11,12] The whole area of all histopathological sections was scanned at ×400 magnification using a light microscope equipped with a high-resolution video camera (Microphoto-FXA, Nikon Co). Approximately 40 images were selected from each heart. Collagen fraction (stained with aniline blue in Masson's trichrome–stained sections) was calculated as the sum of total areas of interstitial fibrosis of the entire field divided by the sum of total connective tissue and myocardial areas of the visual field of the section. Areas of perivascular fibrosis were excluded from this measurement.

For areas of perivascular fibrosis, short-axis images of intramyocardial coronary arteries from 50 to 200 μm in diameter were studied.[11,12] The outer border of the tunica media was traced in each arterial image with Masson's trichrome staining at ×100 to ×200 magnification. Areas encircled by the tracings were calculated. During the quantification procedure, nonround vessels due to oblique transsection or branching were excluded, and only round vessels were studied. The areas of fibrosis (collagen deposition stained with aniline blue) immediately surrounding the blood vessels were then calculated. Perivascular fibrosis was determined as the ratio of the area of fibrosis surrounding the vessel wall to the total vessel area. In each heart, approximately 40 arteries were examined. Average values for each size of vessel were used for analysis.

Immunohistochemistry

Antigenic properties are well preserved and specific immunostaining for TGF-β is enhanced with the methacarn fixation.[33] Paraffin slices 5 μm thick were preincubated with 3% skim milk to decrease nonspecific binding. Sections were incubated overnight at 4°C with the anti-human TGF-β antibody (10 μg/mL; AB-100NA, R&D System),[33] an anti-human TGF-β latency-associated peptide antibody (10 μg/mL; AB-246NA, R & D System),[34] and nonimmune IgG (Zymed Laboratory Inc). Biotinylated and affinity-purified goat anti-rabbit IgG (Nitirei) was used as the secondary antibody. Avidin-biotin amplication was followed by incubation with the substrate 3′,3′-diaminobenzidine. As a final step, tissue samples were counterstained with hematoxylin.

Experimental Protocol 3

In this protocol, to clarify the role of TGF-β on the synthesis of ECM proteins, the effects of an antibody against TGF-β (AB-100NA, R&D System) on left ventricular mRNA levels for TGF-β_1, fibronectin, and type 1 collagen were examined. This antibody is shown to be specific for human TGF-β and also to react with rat TGF-β.[33] Three groups of rats were studied. The control group (n=4) received normal diet and normal drinking water. The second group (L+TGF-βAb, n=4) received L-NAME in its drinking water and the anti–TGF-β antibody (intraperitoneal injection at 1 mg/kg per day). The third group (L+IgG, n=4) received L-NAME in its drinking water and nonimmune IgG (intraperitoneal injection at 1 mg/kg per day). Three days after the treatment, animals were killed and cardiac mRNA levels were determined as in protocol 1.

The ability of the anti–TGF-β antibody to neutralize TGF-β_1 was assayed by [^{3}H]thymidine incorporation on mink lung epithelial cell line Mv1Lu (American Type Culture Collection). Mv1Lu cells in 24-well plates were incubated for 18 hours with 2.5 ng/mL recombinant human and rat TGF-β_1 (a gift from Kirin Brewery Co, Tokyo, Japan) and various concentrations of the TGF-β antibody. Addition of the anti–TGF-β antibody, but not of control IgG, to the MV1Lu cells completely abolished the suppression of [^{3}H]thymidine incorporation induced by recombinant TGF-β_1 (data not shown).

Hemodynamic Parameters and Body Weight

Treatment	Systolic Blood Pressure, mm Hg	Heart Rate, bpm	Body Weight, g
Control			
Day 0	133±4	402±11	341±8
Day 3	132±4	400±10	344±6
Week 1	135±4	402±8	349±10
Week 8	132±3	403±12	371±10‡
L-NAME			
Day 0	131±4	402±8	345±5
Day 3	146±6*	331±21†‡	341±8
Week 1	168±7†‡	341±14†‡	345±12
Week 8	190±8†‡	392±20	316±8*‡
L-NAME + CS-866			
Day 0	131±5	403±18	340±5
Day 3	133±5	403±8	338±6
Week 1	134±5	407±8	339±10
Week 8	133±2	399±6	321±12*
L-NAME+hydralazine			
Day 0	133±6	401±15	344±4
Day 3	134±4	397±7	339±6
Week 1	134±3	402±12	340±6
Week 8	135±3	399±6	318±16*‡
L-NAME+L-arginine			
Day 0	136±3	392±24	342±16
Day 3	149±2*‡	336±28*‡	328±24
Week 1	162±9*‡	342±24*‡	320±28
Week 8	152±8*‡	390±32	326±18*

Values are mean±SE.
*$P<0.05$, †$P<0.01$ vs control group; ‡$P<0.05$ vs day 0.

Statistical Analysis

Data are expressed as mean±SE. Serial time-related changes in parameters of a group were compared by 1-way ANOVA and Bonferroni's multiple comparison test. Differences between groups were determined using 2-way ANOVA and a multiple comparison test. A P value of 0.05 or less was considered statistically significant.

Results

Blood Pressure, Heart Rate, and Body Weight

Changes in the systolic arterial pressure are shown in the Table. The L group showed a progressive rise in systolic arterial pressure. Increases in systolic arterial pressure were similar between the L+L-arg group and the L group. The systolic arterial pressure showed no significant change in the control, L+CS-866, or L+Hyd groups. The L and L+L-arg groups showed a transient reduction in heart rate on the third day and first week. Heart rates were similar in the control, L+Hyd, and L+CS-866 groups throughout the treatment.

Body weights did not differ significantly among the groups before treatment (Table). During the study, rats in the control group gained weight, whereas all other groups treated with L-NAME lost weight in the eighth week.

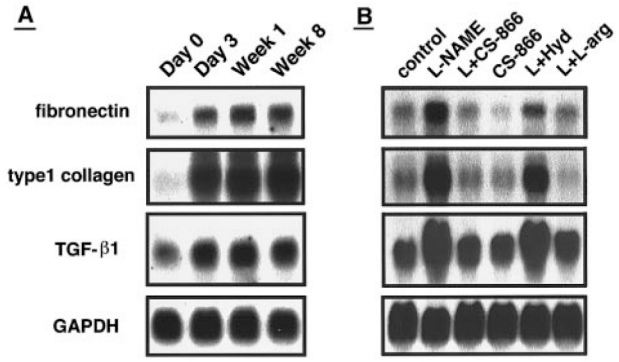

Figure 2. Typical autoradiograms of Northern blot analysis of left ventricular mRNAs for TGF-β, fibronectin, type 1 collagen, and GAPDH on day 0, day 3, week 1, and week 8 of L-NAME administration (A) and after the first week of L-NAME administration (B). The first lane indicates the control group; second lane, L-NAME group; third lane, L-NAME+CS-866 group; fourth lane, CS-866 group; fifth lane, L-NAME+hydralazine group; and sixth lane, L-NAME+L-arginine group.

Cardiac mRNA Levels for TGF-β_1 and ECM Protein (Protocol 1)

The cardiac mRNA levels for TGF-β_1, fibronectin, and type 1 collagen on the third day, first week, and eighth week of L-NAME administration were significantly higher in the L group than in the control group (Figures 2 and 3). The sustained increase in gene expressions was significantly reduced by treatment with the AT_1 receptor antagonist as well as by L-arginine but not by hydralazine (Figures 2 and 3). Treatment with the AT_1 receptor antagonist or L-arginine alone did not affect the mRNA levels (data not shown).

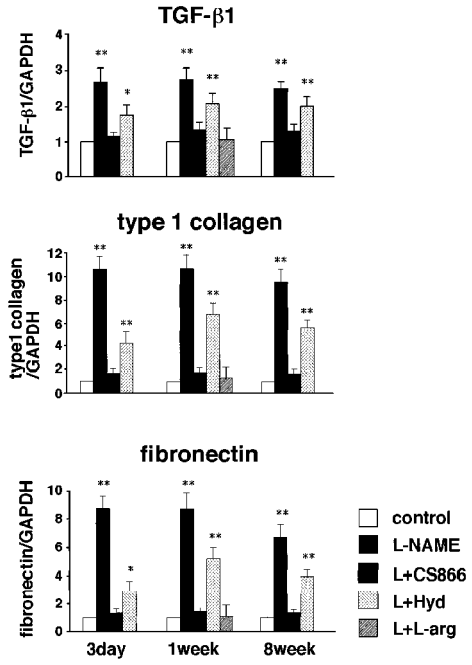

Figure 3. Summary of densitometric analysis. Data are expressed as a ratio of designated mRNA to GAPDH mRNA relative to the control, which was given an arbitrary value of 1. Each group consists of at least 7 rats. Data are expressed as mean±SE. *$P<0.05$, **$P<0.01$ vs control.

Morphometry and Immunohistochemistry (Protocol 2)

In the control group, immunoreactivity for TGF-β and TGF-β latency-associated peptide was constitutively present in areas that normally contain collagen, such as the perivascular and myocardial interstitial spaces, in the control group (data not shown).

At the eighth week of treatment, significant increases in perivascular fibrosis (Figure 4A and 4E) and myocardial interstitial fibrosis were noted in the L group. The vascular and myocardial fibrotic changes were patchily distributed in the left and right ventricles (data not shown). These 2 types of fibrosis were significantly reduced by treatment with the AT_1 receptor antagonist as well as by L-arginine (Figure 5). Treatment with hydralazine had no effect on the cardiac fibrosis induced by L-NAME. In the L group, intense immunoreactivity for TGF-β (Figure 4B and 4F) and TGF-β latency-associated peptide (Figure 4C and 4G) was noted in the area of the fibrotic lesions. No immunoreactivity was observed when the antibody was replaced with nonimmune IgG, which served as a negative control (Figure 4C and 4H).

Effects of Neutralizing Antibody Against TGF-β (Protocol 3)

The cardiac mRNA levels for TGF-β_1, fibronectin, and type 1 collagen were significantly greater in the L+IgG group than in the control group (Figure 6, top). The increases in gene expression of fibronectin and type 1 collagen were significantly reduced by treatment with the anti–TGF-β antibody (Figure 6, bottom). The level of TGF-β_1 mRNA was unaffected by the anti–TGF-β antibody. Neither anti–TGF-β antibody nor control IgG affected the increase in systolic arterial pressure induced by L-NAME (135±4 mm Hg in control, 155±6 mm Hg in L+TGF-βAb, and 156±6 mm Hg in L+IgG groups).

Discussion

TGF-β has been implicated as a major stimulator of the production of ECM proteins, including collagen and fibronectin.[18–24] The present study evaluated the temporal relationship between cardiac fibrosis and the levels of mRNA for TGF-β_1 and ECM proteins in our rat model. We found that the increases in mRNA levels of TGF-β and ECM proteins were evident as early as the third day and were sustained at the first and eighth weeks of L-NAME administration. In contrast, cardiac fibrosis became apparent at the eighth week but not at the first week of L-NAME administration. Thus, the upregulation of the genes of TGF-β_1 and ECM proteins preceded the development of cardiac fibrosis.

We also examined the site of TGF-β production using immunohistochemical methods and found that the areas of fibrosis at the eighth week of L-NAME administration were characterized by increased immunoreactivity for TGF-β and TGF-β latency-associated peptide. The former antibody used in this study recognizes both extracellular and intracellular TGF-β,[33] and the latter antibody recognizes intracellular TGF-β precursor.[30] Thus, the cells stained with both antibodies are thought to be producing TGF-β. However, because the antibody used to detect TGF-β in this study recognizes 3 TGF-β isoforms, we cannot exclude a contribution of TGF-β_2 or TGF-β_3 to this immunoreactivity. The immunohistochem-

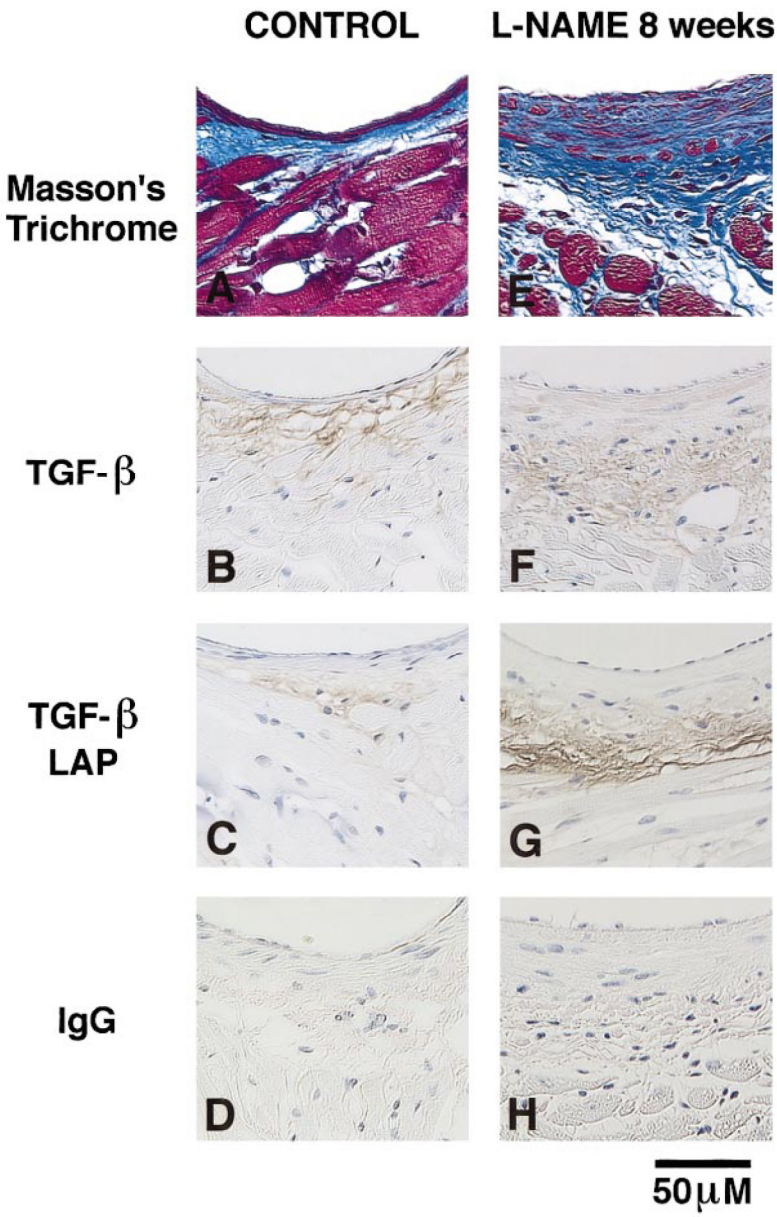

Figure 4. Immunohistochemical localization of TGF-β in coronary arteries in the control group (A, B, C, and D) and the L group at the eighth week (E, F, G, and H). A through D, Adjacent sections from the control group. E through H, Adjacent sections from the L group. A and E, Coronary artery sections stained with Masson's trichrome. B and F, Sections of immunohistochemical staining with antibody to TGF-β. C and G, Sections of immunohistochemical staining with antibody to TGF-β_1 latency-associated peptide (LAP). D and H, Sections of immunohistochemical staining with nonimmune IgG (negative control). Bar indicates 20 μm.

ical findings therefore suggest that substantial amounts of TGF-β protein may be produced by either the activated fibroblast/fibroblast-like cells within the fibrotic lesions. It is unlikely that this cardiac fibrosis and upregulation of the genes were caused by a nonspecific action of L-NAME because the addition of L-arginine, which counteracts the inhibitory effect of L-NAME on NO synthesis, completely inhibited the L-NAME–induced changes in this model. These findings suggest that the enhanced gene expression of TGF-β_1 and ECM proteins contributed to the development of cardiac fibrosis observed in this rat model in vivo.

We have recently shown that the vascular remodeling seen after the long-term administration of L-NAME is prevented by ACE inhibitors[12] as well as by AT_1 receptor antagonists.[35] We therefore examined the effect of the AT_1 receptor antagonist on the cardiac gene expression of TGF-β_1 and ECM proteins in this model. We found that the increases in mRNA levels of TGF-β_1 and ECM proteins and in cardiac fibrosis were markedly reduced by the AT_1 receptor antagonist but not by hydralazine. Thus, the increased activity of angiotensin II via AT_1 receptors, not the arterial hypertension induced by L-NAME, was responsible for the upregulation of the gene expression of TGF-β_1 and ECM proteins in this model. Our conclusion agrees with prior observations that infusion of angiotensin II in rats in vivo caused cardiac fibrosis with TGF-β expression[36,37] and that the upregulation of cardiac TGF-β_1 expression during the process of cardiac fibrosis/hypertrophy[38] in vivo was prevented by an AT_1 receptor antagonist.

Many cell types secrete TGF-β as a latent form that is activated to yield biologically active or mature TGF-β.[30] The precise mechanisms of latent TGF-β activation in vivo are still unknown. Our study with anti–TGF-β antibody suggested that mature TGF-β was generated from the latent complex in the fibrotic tissue, which in turn stimulated gene expression of ECM proteins in this model of cardiac fibrosis. Angiotensin II has been shown to stimulate production of ECM protein through the activation of latent TGF-β in renal mesangial cells in vitro.[28]

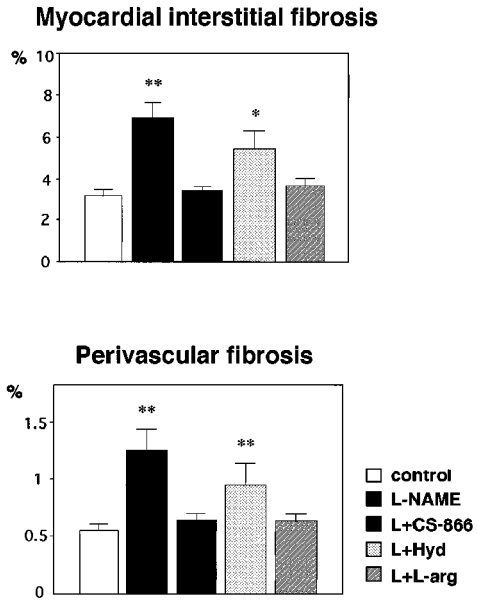

Figure 5. Myocardial interstitial fibrosis (top) and perivascular fibrosis (bottom) of the left ventricle at the eighth week of treatment. Data are expressed as mean±SE. *$P<0.05$, **$P<0.01$ vs control.

Taken together, the findings of the present study suggest the possibility that angiotensin II may be involved in upregulating cardiac TGF-β gene expression and in converting latent TGF-β to the active form in vivo, which is important for the development of cardiac fibrosis in this model.

The activation of the local renin-angiotensin system is assumed to be implicated as the cause of vascular and myocardial remodeling induced by inhibition of NO synthesis. We have previously reported that cardiovascular tissue ACE activity, but not serum ACE activity, increases as early as the first week, while cardiovascular structural changes such as fibrosis develop by the fourth week of L-NAME administration.[12] We also reported that the increase in the number of cardiac AT_1 receptors was evident by the first week of L-NAME administration.[13] Plasma renin activity does not increase at all by the fourth week of L-NAME administration.[12] Both ACE inhibition and AT_1 receptor blockade prevented vascular and myocardial structural changes seen after the eighth week of L-NAME administration.[35] These observations support the hypothesis that a defect in endothelial NO synthesis may lead to the activation of the local renin-angiotensin system, which in turn may contribute to the vascular and myocardial remodeling seen in this model. Recently, we found in preliminary experiments that inhibition of NO synthesis induced early infiltration of monocytes and expression of monocyte chemoattractant protein-1 in vascular and interstitial areas in rat hearts.[39] Because the local renin-angiotensin system is assumed to be activated by tissue fibrosis and inflammation,[40–42] it is possible that these inflammatory changes may account for the early activation of the local renin-angiotensin system.

There are other possibilities that blockade of NO synthesis would cause cardiac fibrosis and inflammation. These include the overexpression of adhesion molecules[43] and chemokines[44] as well as the activation of intracellular reactive oxygen species[45]

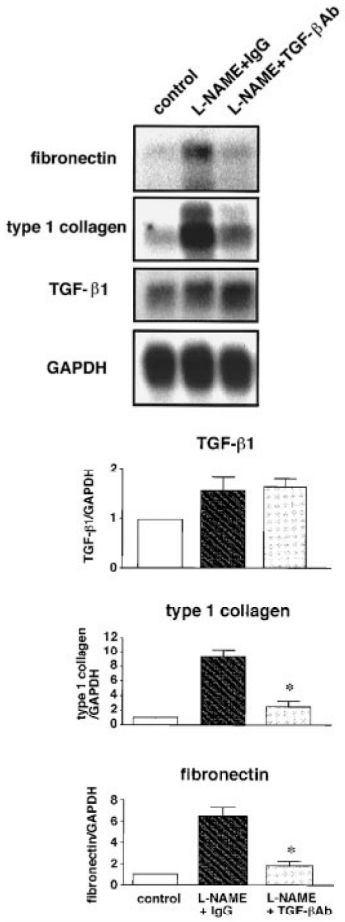

Figure 6. Top, Effect of TGF-β antibody; typical autoradiograms of Northern blot analysis of left ventricular mRNAs for TGF-β, fibronectin, type 1 collagen, and GAPDH on the third day of L-NAME administration. Bottom, Summary of densitometric analysis of data. Data are expressed as a ratio of designated mRNA to GAPDH mRNA relative to the control, which was given an arbitrary value of 1. *$P<0.01$ vs L-NAME+IgG.

and redox-sensitive transcriptional factors.[46] Further studies are needed to elucidate the mechanisms responsible for the fibroinflammatory changes induced by blockade of NO synthesis.

In summary, we demonstrated that the enhanced gene expression and protein production of TGF-β_1 via AT_1 receptors play a key role in the pathogenesis of cardiac fibrosis in the rat model of chronic inhibition of NO synthesis. The present findings suggest that once the intracardiac renin-angiotensin system is activated in certain pathological conditions, the expression of TGF-β induced by angiotensin II causes the accumulation of ECM proteins, which in turn contributes to the development and progression of cardiac fibrosis.

Acknowledgments

This study was supported by grants-in-aid for scientific research (06670725, 06404034, and 07557346) from the Ministry of Education, Science, and Culture, Tokyo, Japan; by the Mitsukosi grant-in-aid (1996) from Mitsukoshi Foundation, Tokyo, Japan; by a research grant from Cell Science Foundation, Osaka, Japan; by a research grant from Suzuken Memorial Foundation, Nagoya, Japan; and by a research grant from Kanae Foudation of Research for New Medicine, Osaka, Japan.

References

1. Moncada S, Higgs A. The L-arginine-nitric oxide pathway. *N Engl J Med.* 1993;329:2002–2012.

2. Dzau V, Gibbons G. Endothelium and growth factors in vascular remodeling of hypertension. *Hypertension*. 1991;24(suppl III):115–121.
3. Cohen RA. The role of nitric oxide and other endothelium-derived vasoactive substances in vascular disease. *Prog Cardiovasc Dis*. 1995;38:105–128.
4. Loscalzo J, Welch G. Nitric oxide and its role in the cardiovascular system. *Prog Cardiovasc Dis*. 1995;38:87–104.
5. Griendling KK, Alexander RW. Endothelial control of the cardiovascular system: recent advances. *FASEB J*. 1996;10:283–292.
6. Egashira K, Inou T, Hirooka Y, Yamada A, Maruoka Y, Kai H, Sugimachi H, Suzuki S, Takeshita A. Impaired coronary blood flow response to acetylcholine in patients with coronary risk factors and proximal atherosclerotic lesions. *J Clin Invest*. 1993;91:29–37.
7. Egashira K, Inou T, Hirooka Y, Kai H, Sugimachi M, Suzuki S, Kuga T, Urabe Y, Takeshita A. Effect of age on endothelium-dependent vasodilation of resistance coronary artery by acetylcholine in humans. *Circulation*. 1993;88:77–81.
8. Egashira K, Suzuki S, Hirooka Y, Kai H, Sugimachi M, Imaizumi T, Takeshita A. Impaired endothelium-dependent vasodilation of large epicardial and resistance coronary arteries in patients with essential hypertension. *Hypertension*. 1995;25:201–206.
9. Zeiher AM, Drexler H, Saubier B, Just H. Endothelium-mediated coronary blood flow modulation in humans: effect of age, atherosclerosis, hypercholesterolemia, and hypertension. *J Clin Invest*. 1993;92:652–662.
10. Ito A, Egashira K, Kadokami T, Fukumoto Y, Takayanagi T, Nakaike R, Kuga T, Sueishi K, Simokawa H, Takeshita A. Chronic inhibition of endothelium-derived nitric oxide synthesis causes coronary microvascular structural changes and hyperreactivity to serotonin in pigs. *Circulation*. 1995;92:2636–2644.
11. Numaguchi K, Egashira K, Takemoto M, Kadokami T, Shimokawa H, Sueishi K, Takeshita A. Chronic inhibition of nitric oxide synthesis causes coronary microvascular remodeling in rats. *Hypertension*. 1995;26(pt 1):957–962.
12. Takemoto M, Egashira K, Usui M, Numaguchi K, Tomita H, Tsutsui H, Shimowaka H, Sueishi K, Takeshita A. Important role of tissue angiotensin-converting enzyme activity in the pathogenesis of coronary vascular and myocardial structural changes induced by long-term blockade of nitric oxide synthesis in rats. *J Clin Invest*. 1997;99:278–287.
13. Katoh M, Egashira K, Usui M, Ichiki T. Cardiac angiotensin II receptor is increased in rats with cardiac remodeling induced by long-term blockade of nitric oxide synthesis. *Circulation*. 1996;94(suppl I):I-656–I-657.
14. Michel JB, Xu Y, Blot S, Philippe M, Chatellier G. Improved survival in rats administered N^G-nitro-L-arginine methyl ester due to converting enzyme inhibition. *J Cardiovasc Pharmacol*. 1996;28:142–148.
15. Hinglais N, Heudes D, Nicoletti A, Mandet C, Laurent M, Bariety J, Michel JP. Colocalization of fibrosis and inflammatory cells in rats. *Lab Invest*. 1994;70:286–294.
16. Nicoletti A, Heudes D, Mandet C, Hinglais N, Bariety J, Michel JP. Inflammatory cells and duration in renovascular hypertensive rats. *Cardiovasc Res*. 1996;32:1096–1107.
17. Nicoletti A, Mandet C, Challah M, Bariety J, Michel JP. Mediators of perivascular inflammation in the left ventricle of renovascular hypertensive rats. *Cardiovasc Res*. 1996;31:585–595.
18. Weber KT, Brilla C. Pathological hypertrophy and cardiac interstitium: fibrosis and renin-angiotensin-aldosterone system. *Circulation*. 1991;83:1849–1865.
19. Isaka Y, Fujiwara Y, Ueda N, Kaneda Y, Kamada T, Imai E. Glomerulosclerosis induced by in vivo transfection of transforming growth factor-β or platelet-derived growth factor gene into the rat kidney. *J Clin Invest*. 1993;92:2597–2601.
20. Sime PJ, Xing Z, Graham FL, Csaky KG, Gauldie J. Adenovirus-mediated gene transfer of active transforming growth factor-β1 induces prolonged severe fibrosis in rat lung. *J Clin Invest*. 1997;100:768–776.
21. Border WA, Nobel NA. Transforming growth factor-β in tissue fibrosis. *N Engl J Med*. 1994;331:1286–1292.
22. Ignotz RA, Massague J. Transforming growth factor-β stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. *J Biol Chem*. 1986;261:4337–4345.
23. Laiho M, Saksela O, Andreasen PA, Keski-Oja J. Enhanced production and extracellular deposition of the endothelial-type plasminogen activator inhibitor in cultured human lung fibroblasts by transforming growth factor-β. *J Cell Biol*. 1986;103:2403–2410.
24. Boluyt OM, O'Neill L, Meredith LA, Bing HLO, Brooks WW, Conrad HC, Crow TM, Lakatta GE. Alterations in cardiac gene expression during the transition from stable hypertrophy to heart failure: marked upregulation of genes encoding extracellular matrix components. *Circ Res*. 1994;75:23–32.
25. Takahashi N, Calderone A, Izzo JN, Marsh DJ, Colucci SW. Hypertrophic stimuli induce transforming growth factor-β1 expression in rat ventricular myocytes. *J Clin Invest*. 1994;94:1470–1476.
26. Villarreal JF, Dillmann HW. Cardiac hypertrophy-induced changes in mRNA levels for TGF-β1, fibronectin and collagen. *Am J Physiol*. 1992;262:H1861–H1866.
27. Sadoshima J, Izumo S. Molecular characterization of angiotensin II–induced hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts. *Circ Res*. 1993;73:413–423.
28. Schorb WG, Booz W, Dostal DE, Conrad KM, Chang KC, Baker KM. Angiotensin II is mitogenic in neonatal rat cardiac fibroblasts. *Circ Res*. 1993;72:1245–1254.
29. Kagami S, Border AW, Millaer ED, Noble AN. Angiotensin II stimulates extracellular matrix protein synthesis through induction of transforming growth factor-β expression in rat glomerular mesangial cells. *J Clin Invest*. 1994;93:2431–2437.
30. Miyazono K, Ichijo H, Heldin CH. Transforming growth factor-β: latent form, binding proteins and receptors. *Growth Factors*. 1993;8:11–22.
31. Mizuno M, Sada T, Ikeda M, Fukuda N, Miyamoto M, Yanagisawa M, Koike H. Pharmacology of CS-866, a novel nonpeptide angiotensin II receptor antagonist. *Eur J Pharmacol*. 1995;285:181–188.
32. Tsuji T, Okada F, Yamaguchi K, Nakamura T. Molecular cloning of the large subunit of transforming growth factor type β masking protein and expression of the mRNA in various rat tissues. *Proc Natl Acad Sci U S A*. 1990;87:8835–8839.
33. Zhang K, Flanders KC, Phan SH. Cellular localization of transforming growth factor-β expression in bleomycin-induced pulmonary fibrosis. *Am J Pathol*. 1995;147:352–361.
34. Basile DP, Rovok JM, Martin DR, Hammerman MR. Increased transforming growth factor-β1 expression in regenerating rat renal tubules following ischemic injury. *Am J Physiol*. 1996;270:F500–F509.
35. Takemoto M, Egashira K, Tomita H, Usui M, Okamoto H, Kitabatake A, Shimokawa H, Sueishi K, Takeshita A. Chronic angiotensin-converting enzyme inhibition and angiotensin II type 1 receptor blockade: effects on cardiovascular remodeling in rats induced by the long-term blockade of nitric oxide synthesis. *Hypertension*. 1997;30:1621–1627.
36. Crawford D, Chobanian A, Brecher P. Angiotensin II induces fibronectin expression associated with cardiac fibrosis in the rat. *Circ Res*. 1994;74:727–739.
37. Kim S, Ohta K, Hamaguchi A, Yukimura T, Miura K, Iwao H. Angiotensin II induces cardiac phenotypic modulation and remodeling in vivo in rats. *Hypertension*. 1995;25:1252–1259.
38. Everett AD, Tufro-McReddie A, Fisher A, Gomez RA. Angiotensin receptor regulates cardiac hypertrophy and transforming growth factor-β_1 expression. *Hypertension*. 1994;23:587–592.
39. Tomita H, Egashira K, Inoue M, Takeya M, Yoshimura T, Takeshita A. Chronic inhibition of nitric oxide synthesis induces monocyte infiltration and myofibroblast formation in the rat heart. *Circulation*. 1997;96(suppl 1):I-492. Abstract.
40. Sun Y, Weber KT. Angiotensin-converting enzyme and myofibroblast during tissue repair in the rat heart. *J Mol Cell Cardiol*. 1996;28:851–858.
41. Sun Y, Weber KT. Cells expressing angiotensin II receptors in fibrous tissue of rat heart. *Cardiovasc Res*. 1996;31:518–525.
42. Campbell SE, Janicki JS, Weber KT. Temporal differences in fibroblast proliferation and phenotype expression in response to chronic administration of angiotensin II or aldosterone. *J Mol Cell Cardiol*. 1995;27:1545–1560.
43. Khan BV, Harrison DG, Olbrych MT, Alexander RW, Medford RM. Nitric oxide regulates vascular cell adhesion molecule 1 gene expression and redox-sensitive transcriptional events in human vascular endothelial cells. *Proc Natl Acad Sci U S A*. 1996;93:9114–9119.
44. Zeiher AM, Fisslthaler B, Schray-Utz B, Busse R. Nitric oxide modulates the expression of monocyte chemoattractant protein 1 in cultured human endothelial cells. *Circ Res*. 1995;76:980–986.
45. Niu XF, Smith CW, Kubes P. Intracellular oxidative stress induced by nitric oxide synthesis inhibition increases endothelial cell adhesion to neutrophils. *Circ Res*. 1994;74:1133–1140.
46. De Caterina R, Libby P, Peng HB, Thannickal VJ, Rajavashisth TB, Gimbrone MA Jr, Shin WS, Liao JK. Nitric oxide decreases cytokine-induced endothelial activation: nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. *J Clin Invest*. 1995;96:60–68.