Role of Transforming Growth Factor-β1 in Cardiovascular Inflammatory Changes Induced by Chronic Inhibition of Nitric Oxide Synthesis

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Abstract—We previously reported that chronic inhibition of nitric oxide (NO) synthesis with Nω-nitro-L-arginine methyl ester (L-NAME) induces inflammatory changes (monocyte infiltration, myofibroblast formation, and monocyte chemoattractant protein-1 [MCP-1] and transforming growth factor-β1 [TGF-β1] expression) in the rat heart and vessel. There is debate regarding whether TGF-β1 exhibits proinflammatory or anti-inflammatory activities. We used the rat model to investigate the role of TGF-β in the pathogenesis of such inflammatory changes. We show here that infiltrating monocytes and myofibroblasts in the inflammatory lesions produced TGF-β1 on the third day of L-NAME administration. Cotreatment with a monoclonal antibody against TGF-β1, but not with control IgG, prevented the L-NAME–induced cardiac inflammation. The antibody also significantly inhibited the gene expression of MCP-1, P-selectin, and intercellular adhesion molecule-1. In summary, the antibody against TGF-β1 prevented inflammatory changes in rat heart and vessel induced by chronic inhibition of NO synthesis, suggesting that increased production of TGF-β1 is involved in the inflammatory changes in this model. (Hypertension. 2000;35:86-90.)

Key Words: endothelium-derived factor ■ growth substances ■ inflammation ■ adhesion molecule ■ angiotensin II ■ fibrosis

Cardiovascular disorders such as hypertension, hypercholesterolemia, atherosclerosis, and aging are associated with endothelial dysfunction that leads to the reduced bioactivity of nitric oxide (NO).1–7 We8–12 and others13 have recently shown that chronic inhibition of NO synthesis with Nω-nitro-L-arginine methyl ester (L-NAME) induces marked monocyte infiltration into the coronary vessels associated with induction of monocyte chemoattractant protein-1 (MCP-1) during the first week of the treatment and causes vascular remodeling (medial thickening and fibrosis) and myocardial remodeling (fibrosis and hypertrophy) after 4 to 8 weeks of the treatment in animals.

We have recently reported that early induction of transforming growth factor-β1 (TGF-β1) contributes to cardiac fibrosis in this model.10 However, the role of TGF-β1 in the pathogenesis of early inflammatory changes in this model has not been examined. There is considerable debate regarding whether TGF-β1 exhibits proinflammatory or anti-inflammatory activities (for review, see References 14 and 15). For example, it has been demonstrated that in vivo gene transfer of TGF-β1 causes tissue inflammatory changes and subsequent fibrosis in the lung16 and blood vessels,17 whereas the major phenotype of the TGF-β1 knockout mice is an early-onset inflammation in most tissues of the body.18,19 TGF-β1 is usually secreted from many cell types as a biologically inactive or latent form.20 The activation mecha-

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1 mg/kg per day). We confirmed that drinking and eating patterns of the rats were unaffected by any treatment protocol. Before and on the third day of treatment, systolic blood pressure (the tail-cuff method), heart rate, and body weight were measured.

We used a monoclonal antibody against TGF-β1 (Biosource, AHG0051) to block TGF-β1 activity in vivo. This antibody was directed against human TGF-β1 and shown to neutralize rat TGF-β1. Previously, we evaluated the ability of the anti-TGF-β1 antibody to neutralize TGF-β1 activity in vitro. In brief, this antibody completely abolished the suppression of [3H]thymidine incorporation induced by recombinant rat TGF-β1 in the Mink Lung epithelial (MV1Lu) cells.

**Histology and Immunohistochemistry**

Five rats in each group were killed on the third day of treatment, as we described previously. In brief, each animal was anesthetized with intraperitoneal pentobarbital, its abdomen was opened, and the abdominal aorta was cannulated. The chest was opened, and an incision was made in the right atrium. 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 for 60 minutes with methacarn solution. The heart was cut into 5 pieces perpendicular to the long axis. All tissue samples were fixed in methacarn solution for a few days, dehydrated, embedded in paraffin, and cut into slices 5 μm thick. Sections were mounted on slides and stained with hematoxylin-eosin solution for estimation of inflammatory cell infiltration.

For immunohistochemistry, paraffin slices 5 μm thick were preincubated with 3% skim milk to decrease nonspecific binding. Sections were incubated overnight at 4°C with mouse anti-rat macrophage/monocyte antibody (ED1, Serotec); mouse anti-human proliferating cell nuclear antigen (PCNA) antibody (Dako); mouse anti-human TGF-β1 antibody (1 μg/mL, AHG0051, Biosource); goat anti-human TGF-β1 latency-associated peptide antibody (10 μg/mL, AB-246NA, R&D); anti-human α-smooth muscle actin antibody (1 μg/mL, Dako), or nonimmune mouse or goat IgG (Zymed Laboratory). The slides were washed and incubated with biotinylated, affinity-purified rabbit anti-mouse or goat IgG as secondary antibody. After avidin-biotin amplification, the slides were incubated with 3',3'-diaminobenzidine and counterstained with hematoxylin.

To determine the cell type of the TGF-β-expressing cells, immunohistochemical double staining was performed. The tissue sections were stained with an antibody against TGF-β and with a monoclonal antibody against α-smooth muscle actin or monocyte.

Morphometry and cell enumeration were performed by a single observer who was blinded to all treatment protocols, as we described. To quantify the number of immunopositive cells in hearts, 5 heart sections per heart immunohistochemically stained by antibodies against ED1 or PCNA were scanned at ×100 magnification. The number of cells positive for ED1 or PCNA was counted, and the sum of total cells per section was reported.

**Northern Blot Analysis**

Five rats in each group were killed on the third day of treatment. After the animals were killed, rat hearts were removed, and the hearts were snap-frozen in liquid nitrogen and stored at −80°C.

Total RNA was extracted from each sample by the acid guai- dinium thiocyanate-phenol-chloroform method, poly(A)+ RNA was purified on an oligo(dT)-cellulose column, and then Northern blot hybridization was performed as we described previously. The cDNA probes used were as follows: a 1.0-kb EcoRI-EcoRI fragment of rat MCP-1 cDNA, a 1.6-kb EcoRI-EcoRI fragment of rat P-selectin cDNA, a 2.6-kb EcoRI-EcoRI fragment of rat intercellular adhesion molecule-1 (ICAM-1) cDNA, and a 1.3-kb PstI-PstI fragment of mouse GAPDH (American Type Culture Collection). Relative amounts of MCP-1, P-selectin, or ICAM-1 mRNA were normalized against the amounts of GAPDH mRNA.

**Statistical Analysis**

Data are expressed as mean±SE. Differences in parameters of a group were compared by Student’s t test. Differences between groups were determined by 2-way ANOVA and a Fisher’s multiple comparison test. A level of P<0.05 was considered statistically significant.

### Results

#### Blood Pressure, Heart Rate, and Body Weight

Changes in systolic arterial pressure are shown in the Table. The L-NAME, L-NAME+IgG, and L-NAME+TGF-βAb groups showed a rise in systolic arterial pressure. Increases in systolic arterial pressure were similar between L-NAME, L-NAME+IgG, and L-NAME+TGF-βAb groups. The L-NAME, L-NAME+IgG, and L-NAME+TGF-βAb groups showed a reduction in heart rate. Body weights did not differ significantly among the groups before and after treatment.

### Localization of TGF-β-Producing Cells

A marked infiltration of mononuclear leukocytes in the perivascular areas immediately surrounding the coronary arteries and veins and the myocardial interstitial spaces was observed in the L-NAME and L-NAME+IgG groups (Figure 1). Attachment of mononuclear leukocytes to the endothelium of coronary vessels was also seen in those 2 groups. The majority of leukocytes that had infiltrated into the lesions were found to be ED1-positive monocytes (Figure 1). Spindle-shaped α-smooth muscle actin–positive cell (myofibroblasts transformed usually from fibroblasts or pericytes) is another major cell type (Figure 1) that had appeared in the inflammatory lesions. Although α-smooth muscle actin is used as a marker for myofibroblasts, the antibody against α-smooth muscle actin also recognizes vascular smooth muscle cells. Therefore, it is possible that these immunopositive cells might have derived from vascular smooth muscle cells that migrated into the perivascular and interstitial inflammatory lesions. Nuclear staining for PCNA antibody was observed in some endothelial cells, vascular smooth muscle cells in the media, monocytes, or myofibroblast-like cells (data not shown). No such inflammatory and proliferative changes were observed in the control group.

Immunoreactivity for TGF-β1 and TGF-β1 latency-associated peptide was weakly present in the areas that...
normally contain collagens, such as the perivascular and myocardial interstitial spaces in the control group (Figure 1). In the L-NAME and L-NAME+IgG groups, both TGF-β1 and TGF-β1 latency-associated peptide immunoreactivity were intensely present in monocytes and/or spindle-shaped fibroblast-like cells (possibly myofibroblasts) (Figure 1). Immunological double staining showed that a considerable proportion of TGF-β1–positive cells were positive for antibodies against both monocytes and α-smooth muscle actin (data not shown).

**Effects of the Anti–TGF-β1 Antibody on Inflammatory and Proliferative Changes**

When ED1-positive monocytes or PCNA-positive cells were counted by use of immunohistochemistry, the number of immunopositive cells per section was significantly greater in the L-NAME and L-NAME+IgG groups than in the control group (Figure 2). The increases in ED1-positive cells and PCNA-positive cells were markedly reduced by treatment with the antibody against TGF-β1. Treatment with the antibody significantly reduced the appearance of α-smooth muscle actin–positive myofibroblasts (data not shown).

**Effects of a Neutralizing Antibody Against TGF-β on mRNA Levels for MCP-1, P-Selectin, and ICAM-1**

The cardiac mRNA levels for MCP-1, P-selectin, and ICAM-1 were significantly higher in the L-NAME and L-NAME+IgG groups than in the control group (Figure 3).
The increases in gene expression of those molecules were significantly reduced by treatment with the anti–TGF-β1 antibody (Figure 3).

**Discussion**

The most important finding of the present study is that the treatment with an antibody against TGF-β1, but not with control IgG, prevented the infiltration of monocytes and myofibroblast formation in the heart and vessel of the rat model with chronic inhibition of NO synthesis. In addition, treatment with the anti–TGF-β1 antibody also inhibited the increase in cells positive for PCNA, a marker of cell proliferation. The observed effect of the antibody was independent of arterial hypertension induced by L-NAME. These results suggest that TGF-β1 is involved in the development of the inflammatory and proliferative changes in this model.

Immunoreactivity for TGF-β1 and TGF-β1 latency-associated peptide was found to be increased in the inflammatory lesions. The former antibody recognizes both extracellular and intracellular TGF-β1, and the latter antibody recognizes intracellular TGF-β1 precursor. Thus, the cells stained with the both antibodies are thought to produce TGF-β1. Our immunohistochemical double staining revealed that infiltrating monocytes and myofibroblasts are the major source of increased production of TGF-β1. Although the activation mechanisms of latent TGF-β1 complex are unclear in the present study, the effect of the antibody suggests that the active form of TGF-β1 was generated from the latent complex, which induced the inflammatory and proliferative changes.

The underlying mechanism of the increase in TGF-β1 production involves increased local activity of angiotensin II via AT1 receptors in this model, because we previously demonstrated that treatment with AT1 receptor antagonist prevented the increases in gene expression of TGF-β1 and extracellular matrix proteins such as type I collagen and fibronectin after L-NAME treatment. Others have also shown that angiotensin II infusion induces TGF-β1 expression in hearts and vessels in vivo. We previously demonstrated that cotreatment with a polyclonal antibody against TGF-β (different from that used in the present study) blocked the increases in gene expression of extracellular matrix proteins, suggesting that the increases in matrix protein production and fibrosis are mediated by TGF-β1. Profibrotic activity of TGF-β1 has been shown to depend on differentiation of interstitial fibroblast and/or pericyte to myofibroblast with acquiring α-smooth muscle actin. Myofibroblasts produce extracellular matrix proteins via autocrine/paracrine action of TGF-β1 and thus are responsible for tissue fibrosis/remodeling. Myofibroblasts are eliminated by apoptosis when the fibrotic process is completed. Although additional studies that examine the effect of long-term suppression of TGF-β1 activity on fibrotic changes would be more interesting, it is recognized that chronic administration of an antibody for longer periods may not be possible. Thus, further studies are needed to more accurately address the notion that TGF-β1 is involved in the development of cardiovascular tissue fibrosis in this model.

Since inflammatory changes in coronary vessels have been reported in animal models with angiotensin II–induced hypertension, genetic hypertension, and renovascular hypertension, inflammatory changes associated with increased production of TGF-β1 seen in our experimental model might result, at least in part, from the rapid increase in systolic arterial pressure induced by L-NAME administration. However, we previously reported that the normalization of hypertension by treatment with hydralazine did not prevent inflammatory changes or upregulation of TGF-β1. Furthermore, in the present study we have shown that treatment with TGF-β1 antibody did not decrease L-NAME–induced hypertension. Others have demonstrated that infusion of angiotensin II at a low dose, which did not produce hypertension, upregulates TGF-β1 expression and induces subsequent fibroinflammatory changes in the rat heart. Thus, it is unlikely that the increase in systolic arterial pressure largely contributed to the induction of inflammatory changes and TGF-β1 expression in our experimental model.

In the present study treatment with anti–TGF-β1 antibody prevented the increased expression of MCP-1, P-selectin, and ICAM-1, suggesting that those inflammation-promoting molecules contribute to inflammatory changes in this model. Under our experimental conditions, it was not possible to discriminate between the primary and secondary actions of
TGF-β1 because the anti–TGF-β1 antibody prevented both inflammatory changes and the gene expression of those inflammation-promoting molecules. The relationship among those molecules such as MCP-1, P-selectin, ICAM-1, and TGF-β1 should be clarified in the future to determine the most important molecule and a therapeutic molecular target for fibroinflammatory disorders in this model.

There are discrepancies between the results presented here and the results of knockout mice for TGF-β1. Mice lacking TGF-β1 develop an early inflammation in multiple organs, including the heart, culminating in death at approximately 3 weeks of age.18,19 Diebold et al.29 recently indicated that such tissue inflammation in the TGF-β1 null mice was secondary to lymphocyte proliferation and activation but was not primarily due to the deficiency of TGF-β1; immunosuppressive interventions blunted the tissue inflammation in the TGF-β1 null mice. Therefore, it appears that such inflammation in TGF-β1 null mice was secondary to lymphocyte proliferation and activation but was not primarily due to the deficiency of TGF-β1; immunosuppressive interventions blunted the tissue inflammation in the TGF-β1 null mice. Therefore, it appears that such inflammation in TGF-β1 null mice resulted, at least in part, from the production of inflammatory cytokines from TGF-β1 null lymphocytes. The notion that TGF-β1 is responsible for chronic fibroinflammatory disorders is supported by prior data that in vivo gene transfer of TGF-β1 causes inflammatory tissue changes and subsequent fibrosis16,17 and that transgenic mice in which mature (biologically active) TGF-β1 is locally expressed in the hepatocytes develop hepatic inflammation and fibrosis.29

In summary, we demonstrated that the increase in TGF-β1 production is of functional importance in the pathogenesis of inflammatory and proliferative changes of hearts and vessels in a rat model of chronic inhibition of NO synthesis. It is suggested that TGF-β1 may be one of the therapeutic molecular targets for fibroinflammatory disorders such as arteriosclerosis or restenotic lesions after angioplasty.

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