Antihypertensive effects of chronic anti-TGF-β antibody therapy in Dahl S rats

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Dahly, Annette J., Kimberly M. Hoagland, Averia K. Flasch, Sharda Jha, Steven R. Ledbetter, and Richard J. Roman. Antihypertensive effects of chronic anti-TGF-β antibody therapy in Dahl S rats. Am J Physiol Regul Integr Comp Physiol 283: R757–R767, 2002. First published May 16, 2002; 10.1152/ajpregu.00098.2002.—This study examined the role of transforming growth factor-β (TGF-β) in the development of hypertension and renal disease in 9-wk-old male Dahl salt-sensitive (Dahl S) rats fed an 8% NaCl diet for 3 wk. The rats received an intraperitoneal injection of a control or an anti-TGF-β antibody (anti-TGF-β Ab) every other day for 2 wk. Mean arterial pressure was significantly lower in Dahl S rats treated with anti-TGF-β Ab (177 ± 3 mmHg, n = 12) than in control rats (190 ± 4 mmHg, n = 17). Anti-TGF-β Ab therapy also reduced proteinuria from 226 ± 20 to 154 ± 16 mg/day. Renal blood flow, cortical blood flow, and creatinine clearance were not significantly different in control and treated rats; however, medullary blood flow was threefold higher in the treated rats than in the controls. Despite the reduction in proteinuria, the degree of glomerulosclerosis and renal hypertrophy was similar in control and anti-TGF-β Ab-treated rats. Renal levels of TGF-β1 and -β2, α-actin, type III collagen, and fibronectin mRNA decreased in rats treated with anti-TGF-β Ab. To examine whether an earlier intervention with anti-TGF-β Ab would confer additional renoprotection, these studies were repeated in a group of 6-wk-old Dahl S rats. Anti-TGF-β Ab therapy significantly reduced blood pressure, proteinuria, and the degree of glomerulosclerosis and renal medullary fibrosis in this group of rats. The results indicate that anti-TGF-β Ab therapy reduces blood pressure, proteinuria, and the renal injury associated with hypertension.

blood pressure; proteinuria; glomerulus; kidney; renal hemodynamics; glomerulosclerosis; transforming growth factor-β

TRANSFORMING GROWTH FACTOR-β (TGF-β) is a multifunctional cytokine with profibrogenic properties that has been implicated in the pathogenesis of renal, cardiac, and vascular end organ damage associated with hypertension and diabetes. TGF-β’s fibrogenic actions result from its ability to simultaneously increase the deposition of extracellular matrix proteins (24), decrease the degradation of matrix proteins (7), and upregulate the expression of integrins, which facilitate matrix assembly (1). Several lines of evidence indicate that TGF-β may play a role in the pathogenesis of renal disease associated with diabetes and hypertension (1, 15, 17, 38, 40). In this regard, circulating and/or local concentrations of TGF-β in the kidney have been reported to be elevated in humans and experimental animals with glomerulonephritis, diabetic nephropathy, and hypertensive glomerular injury (1). Moreover, transgenic animals that overexpress TGF-β develop glomerular lesions and tubulointerstitial renal disease that resemble the types of lesions seen in patients with diabetes or hypertension (2, 17–19, 24).

There is also evidence that the renal production of TGF-β may be stimulated by elevations in dietary salt intake. This may have clinical implications and contribute to the renal, cardiac, and vascular damage that accompanies the development of salt-sensitive forms of hypertension. In this regard, Ying and Sanders (39) reported that elevations in dietary salt intake increase TGF-β1, -β2, and -β3 mRNA levels in the kidneys of Sprague-Dawley rats. Other investigators found that a high-salt diet increases the levels of TGF-β mRNA and protein in the kidneys and the heart of spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats and that is associated with cardiac and renal hypertrophy and fibrosis (40). Similarly, in Dahl salt-sensitive (Dahl S) rats, which rapidly develop severe proteinuria and glomerulosclerosis when fed a high-salt diet (3, 32, 35), Tamaki and coworkers (36) reported that the renal levels of TGF-β mRNA are markedly increased. However, the contribution of TGF-β to the development of hypertension-induced renal disease remains to be established, because no studies have examined the effects of chronic blockade of the production of TGF-β in any model of hypertension. Part of the problem has been due to the lack of inhibitors or molecular approaches to effectively block this pathway.

Recently, Han et al. (11) and Ziyadeh et al. (41) reported that knockdown of the production of TGF-β with antisense TGF-β1 oligodeoxynucleotides or blockade of the actions of TGF-β with a neutralizing Ab prevented the overexpression of TGF-β, the increase in

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urinary microalbumin excretion, and the degree of
glomerulosclerosis and the mesangial matrix expan-
sion in the glomerulus of diabetic db/db mice. The
purpose of the present study was to use a similar
approach to examine the effects of chronic administra-
tion of a murine monoclonal antibody 1D11 that neu-
ralizes all isoforms of TGF-β (5) on the development of
hypertension, proteinuria, glomerulosclerosis, and tu-
bulointerstitial disease in Dahl S rats fed a high-salt
(8.0% NaCl) diet.

METHODS

General Methods

Experiments were performed on male Dahl salt-sensitive
SS/Jr (Dahl S) rats obtained from a colony maintained at the
Medical College of Wisconsin. The rats were housed in an
American Association for Accreditation of Laboratory Animal
Care-approved animal care facility at the Medical College of
Wisconsin, and all protocols were approved by the Medical
College of Wisconsin’s Institutional Animal Care and Use
Committee. The rats were fed a low-salt diet (0.1% NaCl)
until the time of the experiment to maintain normal blood
pressure and minimize renal injury. Water was allowed ad
libitum throughout the study. When rats were 9 wk of age
(250–300 g), they were switched to a high-salt diet (8% NaCl)
for 3 wk. Experiments were performed using four groups of
rats. After 1 wk on the high-salt diet, one group of rats
received an intraperitoneal injection of a murine anti-TGF-β
monoclonal Ab (1D11) at a dose of 5.0 mg/kg every other day
for 2 wk. The second group received a lower dose (0.50 mg/kg)
of the anti-TGF-β Ab every other day for 2 wk. The third
group served as the controls and received an intraperitoneal
injection of an isotype-matched control murine monoclonal
antibody (13C4; antivertoxin) for 2 wk. A fourth group of
rats was maintained on the low-salt diet (0.4% NaCl)
throughout the study so that the degree of baseline renal
damage in age-matched nonhypertensive Dahl S rats could
be determined. The anti-TGF-β Ab (1D11) that was used in
the present study neutralizes all three isoforms of TGF-β (5)
and has a circulating half-life of 15.2 h in rats.

Protocol 1: Effect of Anti-TGF-β Ab Therapy on Blood
Pressure and Renal Function

Measurement of blood pressure in conscious animals. Dur-
ing the second week on the high-salt diet, the rats were
anesthetized with an intramuscular injection of ketamine (40
mg/kg), xylazine (2.5 mg/kg), and acepromazine (0.6 mg/kg).
An indwelling catheter was inserted into the femoral artery
for continuous measurement of mean arterial pressure
(MAP). The catheter was tunneled subcutaneously to the
back of the neck, fed through a Dacron-mesh button sutured
beneath the skin, and advanced through a stainless steel
spring that was connected to a swivel (Instech Laboratories:
Plymouth Meeting, PA) mounted above the animal’s cage.
The rats were allowed 1 wk to recover from surgery. Then,
MAP and heart rate (HR) were recorded at a sample rate of
300 Hz between 1:00 and 5:00 PM on four consecutive
days while the rats were conscious in their home cages. Systolic,
diastolic, and MAP were averaged over 1-min periods and
converted to a mean value for the recording session. The
daily averages of MAP for each animal were reduced to a
single value for the 4-day recording period. After the last
blood pressure recording session, an overnight urine sample
and a blood sample were collected for measurement of pro-
teinuria and urinary creatinine clearance. The protein con-
centration of the urine samples was determined using the
Bradford method (Bio-Rad Laboratories, Hercules, CA).

Measurement of renal hemodynamics in anesthetized ani-
mals. At the end of the chronic study, the rats were anesthe-
tized with an intramuscular injection of ketamine (30 mg/kg)
and an intraperitoneal injection of thioptabarbitol (Inactin,
50 mg/kg). The rats were placed on a thermostatically con-
trolled warming table to maintain body temperature at 37°C.
After rats were tracheotomized, catheters were inserted into
the external jugular vein for intravenous infusions and the
femoral artery for measurement of MAP. A 2-mm flow probe
was positioned around the left renal artery to measure renal
blood flow (RBF) using an electromagnetic flowmeter (Caro-
lina Instruments, King, NC). The rats received an intrave-
nous infusion of a 0.9% NaCl solution containing 1% BSA at
a rate of 6 ml/h throughout the experiment to replace fluid
losses. After a 30-min stabilization period, cortical blood flow
(CBF) was measured from five different sites on the renal
cortex using an external probe (PF-316) and a laser-Doppler
flowmeter (PF3, Perimed, Stockholm, Sweden). Medullary
blood flow (MBF) was measured using an acutely implanted
fiber optic probe, as we have previously described (13).

Histological evaluation of kidneys. At the end of the acute
experiment, the kidneys were rapidly removed without oc-
cluding the blood supply to prevent capillary collapse, and
kidney weights were recorded. The right kidney was frozen in
liquid nitrogen and stored at −80°C for measurement of the
levels of fibronectin, type III collagen, and TGF-β1 and
TGF-β2 mRNA levels using RNase protection assays (RPAs).
The left kidney was hemisected, rinsed in ice-cold saline to
remove blood, and immersion fixed in a 5% buffered formalin
solution. The fixed kidneys were later embedded in paraffin,
sectioned, and stained with both periodic acid-Schiff (PAS)
and Mason’s trichrome stains for light microscopy. Glomer-
ular diameters were measured using a video microscopy
system, and the degree of matrix expansion and glomerular
injury was assessed on a minimum of 20–40 glomeruli/sec-
tion as originally described by Raij et al. (30). The degree
of sclerosis was scored on a 0–4 scale based on the percentage
of glomerular capillary area replaced with extracellular matrix.
A glomerular sclerosis score of two indicates that 50% of
glomerular capillary area is filled in with matrix, whereas a
score of four indicates complete closure of all capillaries
within a given glomerulus (30). To prevent sampling bias
since glomerular injury is regional in the kidney of Dahl S
rats, we systematically scored every glomeruli found within 2
mm of the cortical surface as the kidney was scanned from the
upper to lower pole of the section. The kidney sections
were also examined for the degree of fibrosis of vasa recta
capillaries and the formation of protein casts in tubules in
the outer medulla. The percentage of medullary area occu-
pied by protein casts was determined using a Metamorph
imaging program on at least 10 regions per kidney section.

RPA for Fibronectin, Type III Collagen, and TGF-β1 and
TGF-β2 mRNA

Preparation of the riboprobes. RPA probe templates were
prepared by RT-PCR of RNA isolated from the kidney using
primers that are complementary to the cDNA sequences of
fibronectin (26), collagen type III (9), TGF-β1 (6), and TGF-β2
(20). The linearized cDNAs were transcribed in vitro using
the Maxiscript kit (Ambion, Austin, TX) according to the
manufacturer’s instructions. T7 polymerase and [32P]CTP
(3,000 Ci/mmol; DuPont-NEN, Boston, MA) were included in
the reaction mixture to generate 32P-labeled riboprobes. The
reaction mixture was incubated for 60 min at 37°C, and the cDNA templates were removed by digestion with 0.5 U RNase-free DNase. Full-length RNA probes were purified from the transcription reaction by electrophoresis on 6% polyacrylamide gel, followed by autoradiography, excision of the bands from the gel, and passive diffusion of the probes into an elution buffer (Maxiscript kit) overnight at 37°C. The activity of the probe was quantified by scintillation counting.

RPAs. RNA from the whole kidney was isolated using the RNAqueous kit (Ambion). RPAs were performed using the HybSpeed RPA kit (Ambion) according to the manufacturer’s instructions. Briefly, radiolabeled antisense RNA probe for fibronectin, collagen III, TGF-β1, and TGF-β2 were combined with 10 μg of total cellular RNA and hybridized. A probe for 18s RNA (Ambion) was also included in the hybridizations to normalize for the amount of RNA added. After hybridization, RNAse A/RNAse T1 mix was added to the reactions to degrade unhybridized RNA. Hybridized RNA was separated from smaller digested fragments by electrophoresis on a polyacrylamide gel and visualized by using a phosphorimager. The intensity of the bands corresponding to protected fibronectin, collagen III, and TGF-β1 and TGF-β2 mRNA fragments was quantified using Mac BAS version 2.4 software. The expression of each gene was corrected by dividing probe specific signal by that obtained for a protected 18s RNA fragment.

Immunohistochemistry. Unstained 3-μm-thick paraffin sections were deparaffinized, hydrated, and treated with hyaluronidase (1 mg/ml sodium acetate buffer, pH 5.5, with 0.85% NaCl) for 30 min at 20°C and then washed with Tris-buffered saline (TBS). Incubation with a nonspecific protein-blocking agent was performed according to the manufacturer’s instructions (Elite Vectastain Kit, Vector Laboratories, Burlingame, CA). The sections were incubated overnight at 4°C with 1 μg/ml primary Ab (monoclonal anti-α-smooth actin, Sigma, St. Louis, MO), washed at room temperature with TBS and incubated with biotinylated anti-mouse immunoglobulin (Vector Laboratories) for 1 h at 20°C. After an extensive wash, the sections were incubated with avidin-biotin-peroxidase complex for 30 min at 20°C and developed according to the manufacturer’s recommendations. The slides were counterstained with hematoxylin and viewed at ×400.

Immunohistochemistry for TGF-β1 and TGF-β2. Unstained 3-μm-thick paraffin sections were deparaffinized and placed in Dako targeting retrieval solution at 95°C for 90 min (Dako Industries, Carpinteria, CA) and then at 20°C for 20 min. Sections were blocked with 1% BSA for 30 min at 20°C. The slides were then incubated with a rabbit polyclonal TGF-β primary Ab 1:200 (Santa Cruz, Santa Cruz, CA) for 90 min at 20°C. Sections were washed with 0.05 M TBS and incubated with a goat-anti-rabbit IgG FITC-conjugated secondary Ab for 60 min at 20°C (1:100, Santa Cruz). The slides were washed with TBS (pH 7.6, 0.5 M) and distilled H2O, counterstained with Evan’s blue (0.002%) for 10 min at 20°C, and viewed at ×400.

Protocol 2: Early Intervention With Anti-TGF-β Ab Therapy

General methods. To determine whether earlier treatment of Dahl S rats with the anti-TGF-β Ab therapy would confer additional renoprotection, experiments were repeated on younger male Dahl S rats that were 6 wk of age (175–200 g) at the start of the study. The rats were divided into three groups. One group received an intraperitoneal injection of anti-TGF-β Ab (1D11) (0.50 mg/kg) every other day for 3 wk, while the control group received an intraperitoneal injection of the antiverotoxin control Ab (13C4). A third group of rats was maintained on a low-salt diet (0.1% NaCl) for the duration of the experiment to determine the baseline degree of glomerulosclerosis in age-matched, nonhypertensive Dahl S rats.

Time course of the development of proteinuria. An overnight control urine sample was collected while the rats were on a low-salt diet (0.1% NaCl). Then, urine samples were collected from the control rats and anti-TGF-β Ab-treated rats on days 4, 11, 18, and 21 after being placed on a high-salt diet (8.0% NaCl). The protein concentration of the urine samples was determined using the Bradford method (Bio-Rad Laboratories). Urinary albumin concentration was determined by the albumin blue 580 method (Molecular Probes).

Measurement of blood pressure. At the end of the 3-wk study, the rats were anesthetized with ketamine (30 mg/kg im) and thiobutabarbital (Inactin, 50 mg/kg ip) and placed on a thermostatically controlled warming table to maintain body temperature at 37°C. After a cannula was placed in the trachea, the rats were ventilated to maintain a P0.5 of 35–40 mmHg. The femoral artery was cannulated and MAP was directly recorded after a 30-min equilibration period. In the first series of experiments, we verified that blood pressures measured in ketamine- and Inactin-anesthetized Dahl S rats were comparable to those measured in the same rats when conscious. After blood pressure was measured, the kidneys were collected, and the degree of tubulointerstitial damage in the outer medulla and glomerular damage were assessed as described above.

Statistics

Mean values ± SE are presented. The significance of differences in mean values measured in control and anti-TGF-β Ab-treated groups was analyzed using an unpaired t-test or an analysis of variance for repeated measures followed by the Duncan’s multiple-range test. A P value <0.05 was considered statistically significant.

RESULTS

Effect of Anti-TGF-β Ab Therapy on Blood Pressure and Proteinuria in Conscious Dahl S Rats

The effect of chronic anti-TGF-β Ab treatment on the development of hypertension in 9-wk-old male Dahl S rats fed a high-salt diet for 3 wk is presented in Fig. 1. There was no significant difference in blood pressure measured in the rats given the low and the high doses of anti-TGF-β Ab; therefore, the data from these two groups were combined. MAP averaged 190 ± 4 mmHg in control Dahl S rats (n = 12) fed a high-salt diet for 3 wk. MAP was significantly lower in Dahl S rats treated with the anti-TGF-β Ab (177 ± 3 mmHg, n = 17).

The effect of anti-TGF-β Ab treatment on the excretion of protein in Dahl S rats is presented in Table 1. Proteinuria fell from 226 ± 20 to 154 ± 16 mg/day in the Dahl S rats treated with the anti-TGF-β Ab. Despite the reduction in the urinary excretion of protein, indexes of glomerular injury such as the plasma creatinine concentration were not significantly different and averaged 0.9 ± 0.2 mg/dl in the control rats and 1.3 ± 0.2 mg/dl in the rats treated with the anti-TGF-β Ab (Table 1). Both values are elevated compared with a
normal value of 0.52 ± 0.06 mg/dl measured in a group of normotensive, salt-resistant Brown Norway rats (n = 16) fed the same diet for 3 wk. Creatinine clearances were not significantly different and averaged 0.40 ± 0.09 ml·min⁻¹·g kidney wt⁻¹ in the control Dahl S rats and 0.35 ± 0.07 ml·min⁻¹·g kidney wt⁻¹ in the rats treated with the anti-TGF-β Ab (Table 1).

Effect of Anti-TGF-β Ab Therapy on Renal Hemodynamics in Anesthetized Dahl S Rats

The effect of anti-TGF-β Ab treatment on renal hemodynamics is presented in Table 2. RBF was not significantly different in the control (3.13 ± 0.67 ml·min⁻¹·g kidney wt⁻¹) and anti-TGF-β Ab-treated rats (3.22 ± 0.41 ml·min⁻¹·g kidney wt⁻¹) (Table 2). There was also no difference in the laser-Doppler cortical blood flow (CBF) signal measured in the control rats (2.26 ± 0.19 V) and anti-TGF-β Ab-treated rats (1.85 ± 0.23 V). On the other hand, the medullary blood flow (MBF) signal was threefold greater in anti-TGF-β Ab-treated rats (0.99 ± 0.12 V) than in the control rats (0.39 ± 0.09 V). The failure to detect a change in cortical or kidney blood flow despite a large increase in MBF is not surprising given that blood flow to the inner medulla only represents a small fraction of the flow to the kidney (<1%).

Table 1. Effect of anti-TGF-β Ab treatment in Dahl S rats fed a high-salt (8.0% NaCl) diet for 3 wk

<table>
<thead>
<tr>
<th>Proteinuria, mg/day</th>
<th>Control</th>
<th>Anti-TGF-β Ab Therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>226 ± 20 (n = 12)</td>
<td>154 ± 16* (n = 20)</td>
</tr>
<tr>
<td>Plasma creatinine concentration, mg/dl</td>
<td>0.9 ± 0.2 (n = 11)</td>
<td>1.3 ± 0.2 (n = 16)</td>
</tr>
<tr>
<td>Creatinine clearance, ml·min⁻¹·g kidney wt⁻¹</td>
<td>0.40 ± 0.09 (n = 7)</td>
<td>0.35 ± 0.07 (n = 8)</td>
</tr>
<tr>
<td>Left kidney wt, g</td>
<td>1.87 ± 0.06 (n = 12)</td>
<td>1.76 ± 0.06 (n = 20)</td>
</tr>
</tbody>
</table>

Values are means ± SE. Numbers in parentheses indicate the number of animals per group. *Significant difference from the value in the control rats.

Kidney weights were similar in control (1.87 ± 0.06 g) and anti-TGF-β Ab-treated (1.76 ± 0.06 g) rats, indicating that the degree of renal hypertrophy was similar in the two groups (Table 1). The effect of blocking the effects of TGF-β on glomerular morphology is illustrated by the representative PAS-stained kidney sections presented in Fig. 2A and B. Histological examination of glomeruli from the control Dahl S rats (Fig. 2A) and anti-TGF-β Ab-treated Dahl S rats (Fig. 2B) indicated that there was marked expansion of the mesangial matrix in nearly every glomerulus examined. A large percentage of glomerular capillaries was filled with matrix material and there was PAS-positive material in most of the injured glomeruli. Treating Dahl S rats with the anti-TGF-β Ab had no effect on mean glomerular diameter (123.3 ± 1.4 µm; n = 140 glomeruli, 7 rats) vs. control rats (121.2 ± 2.3 µm; n = 176 glomeruli, 8 rats). Chronic treatment of rats with the anti-TGF-β Ab also had no significant effect on the degree of glomerular injury. Focal glomerulosclerosis scores averaged 2.5 ± 0.11 (n = 140 glomeruli, 7 rats) (63% damage) in the control rats vs. 2.8 ± 0.18 (n = 176 glomeruli, 8 rats) (70% damage) in the rats treated with the anti-TGF-β Ab therapy. We also examined the degree of sclerotic seen in a group of normotensive Dahl S rats maintained on a low-salt diet throughout the study (0.4% NaCl). The glomerular injury score (2.5 ± 0.06; n = 188 glomeruli, 8 rats) observed in these rats was not significantly different from that seen in Dahl S rats fed the high-salt diet. These results are consistent with previous reports that Dahl S rats exhibit a high degree of glomerular damage even when maintained on a low-salt diet to minimize the development of hypertension (35).

A comparison of the appearance of the outer medulla of the kidney of control (Fig. 2C) and anti-TGF-β Ab-treated rats (Fig. 2D) is presented in Fig. 2. In the
Untreated Dahl S rats, there is increased deposition of connective tissue in the vasa recta bundles, necrosis of the thick ascending limbs, and the formation of protein casts in the outer medulla (Fig. 2C). In the anti-TGF-β Ab-treated Dahl S rats (Fig. 2D), the number of patent vasa recta capillaries was significantly increased compared with levels seen in the control Dahl S rats and the degree of necrosis of thick ascending limbs and formation of protein casts in the outer medulla was markedly reduced.

Immunohistochemical staining for α-smooth muscle actin, a marker for myofibroblasts associated with pathologic fibrosis (28), was observed in Bowman’s capsule, tubular epithelial cells, mesangial cells, and the renal cortical interstitium in control Dahl S rats (Fig. 3A). Heavy positive staining for smooth muscle actin was also observed in the interstitium and vasa recta vascular bundles in the outer medulla of these animals (Fig. 3C). Rats treated with anti-TGF-β Ab exhibited substantially less staining for α-smooth muscle actin in cortical and medullary regions of the kidney (Fig. 3, B and D). Nearly all of the staining was restricted to the wall of renal arteries, which is consistent with the typical pattern of staining for α-smooth muscle actin seen in normotensive strains of rats.

A comparison of the levels of TGF-β1 and TGF-β2 mRNA expressed in the kidneys of control and anti-TGF-β Ab-treated rats is presented in Fig. 4. The expression of TGF-β1 and TGF-β2 mRNA was markedly reduced in kidneys of Dahl S rats treated with the anti-TGF-β Ab. This finding is consistent with the lower levels of mRNA encoding type III collagen and the extra domain A (EDA)-containing variant of fibronectin seen in the kidney of anti-TGF-β Ab-treated rats compared with the levels seen in control Dahl S rats (Fig. 5).

The results of our immunohistochemistry studies indicate that there are elevated levels of TGF-β1 protein expressed in the glomeruli, proximal tubules, and interstitial space of Dahl S rats fed a high-salt diet (Fig. 6A). The intense staining in the interstitial space around some glomeruli may be due to the loss of integrity of the Bowman’s space and accumulation of filtrate into the interstitium. The anti-TGF-β Ab treatment markedly reduced the staining for TGF-β1 protein in both the renal cortical and medullary interstitium and in the glomerulus (Fig. 6B). Similar results were obtained using anti-TGF-β2 primary Ab.

Protocol 2: Early Intervention With Anti-TGF-β Ab

Proteinuria and microalbuminuria. The effect of anti-TGF-β Ab treatment on the excretion of protein in Dahl S rats fed a high-salt diet is presented in Fig. 7A.
Fig. 3. Immunohistochemical staining for α-smooth muscle actin, a myofibroblast marker. Dahl S rats treated with the control Ab exhibited significant staining in both cortical (A) and medullary (B) regions. Staining was especially prominent surrounding vascular structures and in tubulointerstitial spaces. Some, but not all, glomeruli showed positive staining in the mesangium. Rats treated with anti-TGF-β Ab exhibited very little staining in cortical regions (C) where it was restricted to vessel walls. The staining for α-smooth muscle actin was substantially reduced in the outer medulla (D) of Dahl S rats treated with anti-TGF-β Ab where light staining was evident in association with the vasa recta.

Fig. 4. Effect of anti-TGF-β Ab therapy on the expression of TGF-β1 (A) and TGF-β2 (B) mRNA in the kidney. Dahl S rats (9 wk old) were fed a high-salt (8% NaCl) diet for 3 wk and given an intraperitoneal injection of an anti-TGF-β Ab (0.5 mg/kg) or a control Ab every other day during the last 14 days of the high-salt diet. Numbers in parentheses indicate the number of animals per group. *Significant difference from the value in the control rats.
Protein excretion averaged <20 mg/day in the anti-TGF-β Ab-treated and control Dahl S rats when fed a low-salt (0.1% NaCl) diet. It gradually rose in both the control and anti-TGF-β Ab-treated rats during the first 2 wk of a high-salt diet. By day 18 of the high-salt diet, severe proteinuria was observed in both experimental groups; however, the degree of proteinuria tended to be lower in the anti-TGF-β Ab-treated group than in the control group (74 ± 12 vs. 103 ± 17 mg/day). After 3 wk on a high-salt diet, the severity of proteinuria in Dahl S rats was significantly reduced from 172 ± 20 mg/day in control rats to 91 ± 20 mg/day in the anti-TGF-β Ab-treated rats.

The effect of anti-TGF-β Ab treatment on the urinary excretion of albumin in Dahl S rats is presented in Fig. 7B. In both the control and anti-TGF-β Ab-treated rats, albumin excretion was <10 mg/day when the rats were fed a low-salt diet (0.1% NaCl). After 3 wk on a high-salt diet, albumin excretion was significantly lower in Dahl S rats treated with anti-TGF-β Ab therapy (45 ± 8 mg/day) than in control rats (85 ± 21 mg/day).

Histological evaluation of kidneys. A comparison of renal injury scores in control and anti-TGF-β Ab-treated rats is presented in Fig. 8. A large percentage of glomerular capillaries was filled with matrix material and there was PAS-positive material in most of the severely injured glomeruli. Chronic treatment of these younger Dahl S rats with anti-TGF-β Ab significantly reduced the degree of glomerular injury. The glomerular injury scores averaged 3.25 ± 0.06 in Dahl S rats treated with the control Ab (n = 144 glomeruli, 7 rats) vs. 2.73 ± 0.04 (n = 382 glomeruli, 15 rats) in the anti-TGF-β Ab-treated Dahl S rats (Fig. 8A) and 2.46 ± 0.05 (n = 122 glomeruli, 6 rats) in Dahl S rats maintained on a low-salt diet throughout the study to prevent hypertension.

Anti-TGF-β Ab therapy also reduced the degree of fibrosis of vasa recta capillaries, necrosis of the thick

Fig. 6. Representative sections illustrating the effect of anti-TGF-β Ab therapy on the expression of TGF-β in the glomerulus of Dahl S rats. Kidney sections were stained with an anti-TGF-β1 primary Ab. In Dahl S rats, there was positive staining for TGF-β1 in the glomerulus, proximal tubules, and interstitial space around the glomerulus (A). In rats treated with anti-TGF-β Ab, there was a marked reduction in staining for TGF-β1 in the proximal tubules and renal interstitium (B). These rats only had a small amount of staining for TGF-β1 in the glomerulus.
ascending loop of Henle, and the formation of protein casts in the outer medulla. In this regard, $22.2 \pm 1.3\%$ of the area in the outer medulla was occupied by protein casts in control rats compared with only $5.6 \pm 0.3\%$ of the outer medulla in rats treated with the anti-TGF-β Ab (Fig. 8B). Dahl S rats maintained on a low-salt diet for life exhibited the same percentage of protein casts in the outer medulla ($2.2 \pm 0.3\%$) as was seen in the rats treated with the anti-TGF-β Ab.

**DISCUSSION**

The present study examined the effects of chronic blockade of the actions of TGF-β with a murine monoclonal Ab (1D11) that neutralizes all three of the isoforms of TGF-β (β1, β2, and β3) on the development of hypertension, glomerulosclerosis, and/or tubulointerstitial renal disease in Dahl S rats fed a high-salt diet for 3 wk. The results indicate that chronic treatment of Dahl S rats with an anti-TGF-β Ab significantly reduces blood pressure, proteinuria, and albuminuria. The mechanism by which anti-TGF-β Ab therapy lowers blood pressure in Dahl S rats remains to be established; however, we did find that there was marked fibrosis of the vasa recta capillary bundles resulting in complete closure of most of the capillaries in the outer medulla of Dahl S rats fed a high-salt diet. This led to severe medullary ischemic injury, tubular necrosis, and the formation of protein casts (Fig. 2). Chronic treatment of the 9-wk-old Dahl S rats with the anti-TGF-β Ab reduced the deposition of matrix in vasa recta bundles, preserved MBF, and reduced the degree of tubular necrosis and the formation of protein casts in the outer medulla. The beneficial effects of anti-TGF-β Ab therapy were even more apparent when we studied the effects of the anti-TGF-β Ab treatment in younger (6 wk of age) Dahl S rats that had less preexisting renal damage. In this group, we found that the area occupied by protein casts was fourfold greater in the outer medulla of the control Dahl S rats than seen
in the anti-TGF-β Ab-treated rats. Consistent with these histological findings, we found that MBF measured using laser-Doppler flowmetry was threefold higher in Dahl S rats treated with the anti-TGF-β Ab than in control rats. These results are consistent with the recent finding that chronic administration of TGF-β to normotensive Sprague-Dawley rats reduces MBF and that this is associated with marked fibrosis of vasa recta capillaries and tubular necrosis in the outer medulla of the kidney (16). Collectively, these findings suggest that the fall in creatinine clearance observed in Dahl S rats fed a high-salt diet may involve fibrosis of vasa recta capillaries, medullary hypoperfusion, hypoxic injury to the thick ascending limb, acute tubular necrosis, the formation of protein casts, and tubular obstruction. Furthermore, our results suggest that chronic treatment of rats with an anti-TGF-β Ab may improve renal function by ameliorating the pathological changes that occur in the outer medulla.

The preservation of MBF may also contribute to the antihypertensive effect of the anti-TGF-β Ab therapy observed in Dahl S rats. In previous studies, we reported that sodium reabsorption in the thick ascending limb is markedly elevated in Dahl S rats (13, 42) and this leads to volume expansion that accounts for the initial rise in blood pressure when Dahl S rats are fed a high-salt diet (10). However, with time, MBF falls in Dahl S rats and this contributes to a further blunting of the pressure-natriuresis relationship (4, 31) and an increase in the severity of the hypertension. Other investigators have reported that chronic renal interstitial infusion of L-arginine to elevate the production of nitric oxide prevents the fall in MBF and attenuates the development of hypertension in Dahl S rats (22, 23). Moreover, reductions in MBF have been linked to the resetting of the pressure-natriuresis relationship and the development of hypertension in many other models of hypertension, including the spontaneously hypertensive rat (4, 14, 32) and N^ω-nitro-L-arginine methyl ester (21) and vasopressin-induced hypertension (25, 27).

On the other hand, numerous investigators have shown that TGF-β1 alters the expression of endothelial nitric oxide synthase and COX-2 and components of the renin-angiotensin system, vascular smooth muscle, and other tissues. Thus it is just as likely that the antihypertensive effect of TGF-β therapy in Dahl S rats may be due to blockade of the actions of TGF-β on the expression of these paracrine factors that regulate vascular tone (8, 12). Thus further work is needed to sort out the relative contributions of changes in renal and vascular function to the fall in blood pressure after anti-TGF-β Ab therapy.

In addition to its effect on blood pressure, we found that treating Dahl S rats with anti-TGF-β Ab therapy reduced protein excretion in Dahl S rats fed a high-salt diet. However, no improvement in the degree of glomerular injury at the light microscopic level was observed in the kidneys of 9-wk-old Dahl S rats chronically treated with the anti-TGF-β Ab. This was an unexpected finding, because the anti-TGF-β Ab therapy greatly reduced expression on the TGF-β1 and TGF-β2 mRNA and protein in the glomerulus and renal interstitium of these rats. There is a large body of evidence correlating changes in TGF-β expression in the glomerulus with the degree of extracellular matrix expansion in the glomerulus of diabetic rats (33), normotensive rats (38), transgenic mice that overexpress TGF-β (18), and Dahl S rats (36). We therefore postulated that, although blockade of TGF-β probably attenuated the hypertension-induced glomerular damage, the treatment could not reverse the high degree of preexisting glomerulosclerosis seen in the kidneys of Dahl S rats maintained on a low-salt diet. To test this hypothesis, we studied the effect of anti-TGF-β Ab therapy in young (6 wk old) Dahl S rats that have less preexisting glomerular injury. After 3 wk on a high-salt diet, the control 6-wk-old Dahl S rats exhibited the same degree of glomerular injury as was seen in the 9-wk-old rats. However, the degree of glomerulosclerosis in the anti-TGF-β Ab-treated group was significantly reduced and not different from the degree of injury seen in age-matched nonhypertensive Dahl S rats maintained on a low-salt diet (0.1% NaCl).

The mechanism by which chronic treatment of Dahl S rats reduces the expression of TGF-β mRNA and protein in the kidney remains to be determined. This may simply reflect the fact that TGF-β production is upregulated in damaged glomeruli and ischemic tubular cells in Dahl S rats and treatment of the animals with the antibody lowers TGF-β production by reducing the degree of hypertension-induced renal injury. Alternatively, TGF-β is known to induce expression of growth factors and components of the renin-angiotensin system that in turn increase the production of TGF-β (37). By neutralizing the actions of TGF-β, the antibody may interrupt this positive-feedback loop.

An important question that remains to be answered is how does anti-TGF-β Ab therapy reduce proteinuria in 9-12 wk-old Dahl S rats without altering the degree of glomerular damage? One possibility is that TGF-β may directly affect the permeability properties of the glomerulus to proteins or alter glomerular hemodynamics. In this regard, Sharma et al. (34) recently demonstrated that TGF-β can directly increase the permeability of isolated glomeruli to albumin and this may contribute to proteinuria in vivo.

In summary, chronic administration of anti-TGF-β Ab lowered blood pressure and decreased urinary excretion of protein and albumin in Dahl S rats fed a high-salt diet for 3 wk. Anti-TGF-β Ab therapy did not alter total RBF or CBF in Dahl S rats. However, MBF was significantly higher in Dahl S rats treated with the anti-TGF-β Ab. This observation, coupled with histological evidence of reduced fibrosis of the vasa recta bundles and tubular necrosis in the outer medulla of the kidney, suggests that the renoprotective effects of anti-TGF-β Ab therapy may involve changes in renal medullary hemodynamics and/or changes in the permeability of glomerular capillaries to albumin. Overall, these findings indicate that anti-TGF-β Ab treatment may have therapeutic potential in reducing protein-
uria and renal injury associated with salt-sensitive hypertension and perhaps diabetes.

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