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HORMONES – CYTOKINES – SIGNALING

Combining TGF-β inhibition and angiotensin II blockade results in enhanced antifibrotic effect

Ling Yu, Wayne A. Border, Ian Anderson, Matthew McCourt, Yufeng Huang, and Nancy A. Noble

Fibrosis Research Laboratory, Division of Nephrology, University of Utah School of Medicine; and Cambridge Antibody Technology, Cambridgeshire, United Kingdom

Combining TGF-β inhibition and angiotensin II blockade results in enhanced antifibrotic effects.

Background. Although angiotensin II (Ang II) blockade is rapidly becoming standard antifibrotic therapy in renal diseases, current data suggest that Ang II blockade alone cannot stop fibrotic disease. New therapies, such as antibodies to transforming growth factor-β (TGF-β), or drug combinations will be required to further slow or halt disease progression. Here, using the anti-Thy1 model of glomerulonephritis, the maximally therapeutic dose of the TGF-β neutralizing mouse monoclonal antibody (1D11) was determined and compared with the maximally effective dose of enalapril. Then, the effect of combining both treatments at maximal doses was determined.

Methods. After disease induction with the anti-Thy1 antibody, OX-7, increasing doses of 1D11 were given intraperitoneally (IP) on days 1, 3, and 5. Enalapril was administered in drinking water from day 1. The fibrotic response was assessed at day 6.

Results. 1D11 dose-dependently reduced fibrosis, with the 0.5 and 5 mg/kg doses showing maximal therapeutic effects, reducing period-acid Schiff (PAS) staining by 56% and 45%, respectively. Fibronectin and collagen I staining was reduced by 32% to 36%, respectively. Glomerular mRNA and production of fibronectin, plasminogen activator inhibitor-1 (PAI-1), TGF-β1, and p-Smad2 protein were also reduced. The maximal therapeutic effects of 1D11 and enalapril alone were very similar. However, combination therapy led to further reduction in disease. Notably, matrix deposition was reduced by 80%.

Conclusion. While 1D11 or enalapril at maximal doses reduce fibrosis equally, simultaneous blockade of Ang II and TGF-β reduces fibrotic disease considerably more, offering hope that such drug combinations may confer a therapeutic advantage over angiotensin blockade alone.

In recent years, the number of patients developing end-stage renal disease (ESRD) has dramatically increased worldwide [1]. Slowing or stopping the progression to ESRD is the biggest challenge facing nephrologists today. Progressive accumulation of extracellular matrix (ECM) in glomeruli and interstitium characterizes chronic renal disease, regardless of the underlying etiology [2, 3]. This points to a final common pathway for ESRD. Identification of key mediators of matrix accumulation and development of strategies to block the action of these mediators is now the focus of many investigators. Two of these key mediators are angiotensin II (Ang II) and transforming growth factor-β (TGF-β).

Blockade of Ang II significantly limits renal matrix expansion in numerous experimental models of acute and chronic renal disease [4], indicating that Ang II is a central effector in the pathogenesis of renal fibrosis. Although angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin II type I receptor antagonists (AT_1RAs) have been remarkably successful in clinical trials [5–12], their full therapeutic potential in humans may not have yet been realized because of inadequate dosing. Nonetheless, from work with animal models [13] and the overall clinical experience, it appears unlikely that these drugs alone will entirely halt fibrosis at any dose. A promising and perhaps necessary approach to further disease reduction is combination of new antifibrotic therapies with inhibitors of the renin-angiotensin system.

Of an increasing number of therapies showing efficacy in animal studies, neutralizing antibodies to TGF-β are the most thoroughly studied. From the first studies published in 1990 demonstrating TGF-β's fibrogenic effects [14–16], overwhelming evidence now exists that sustained over-expression of TGF-β is a key mediator of fibrotic disease. Its fibrogenic actions include: (*1*) stimulation of matrix synthesis; (*2*) inhibition of matrix degradation; and (*3*) modulation of matrix receptor expression to facilitate cell-matrix interactions. Many strategies to block TGF-β have been used in animal studies. TGF-β antibodies, antisense oligonucleotides [17, 18], soluble TGF-β type II receptor [19] or type III receptor [20], the TGF-β inhibitor decorin [21, 22], TGF-β's latency-associated peptide [23],

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the negative regulatory signaling molecule Smad7 [24], and inhibition of TGF-β activation [25] have all shown therapeutic efficacy. Among these strategies, TGF-β antibodies are the best developed and most thoroughly studied. Many in vivo studies have demonstrated their antifibrotic effects in renal fibrosis [16, 26–30], pulmonary fibrosis [31], arterial restenosis [32], and skin scarring and thickening [33–35]. Long-term TGF-β antibody treatment has so far been shown to be safe and to effectively prevent glomerulosclerosis in a mouse model of type 2 diabetes [27]. In addition, advances in antibody technology now make production of totally human antibodies a reality, enhancing their clinical utility as therapeutics. To date, however, there are no animal experiments designed to answer three important questions: how much disease reduction is possible with these antibodies; how does this disease reduction compare side-by-side with Ang II blockade alone; and can disease reduction be increased if maximally effective doses of both drugs are combined.

Here we used the model of acute glomerulonephritis induced by injection of an anti-Thy1 antibody to investigate the potential of different doses of the mouse anti–TGF-β1, -β2, and -β3 monoclonal antibody, 1D11, to reduce disease. We then compared the maximally effective dose of 1D11 with the previously determined maximally effective dose of the ACEI, enalapril. Finally, we determined whether combination of the antibody and enalapril at maximally therapeutic doses could further reduce disease.

METHODS

Materials

The anti–TGF-β antibody, 1D11, which neutralizes isoforms TGF-β1, -β2, and -β3, was kindly provided by Cambridge Antibody Technology (Granta Park; Cambridgeshire, UK) and Genzyme Corporation (Cambridge, MA, USA).

Unless otherwise indicated, materials, chemicals, or culture media were purchased from Sigma Chemical Co (St. Louis, MO, USA).

Animals

The studies were performed on male Sprague Dawley rats (200 to 250 g) obtained from the Sasco colony of Charles River Laboratories (Wilmington, MA, USA). Animal housing and care were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, NIH publication no. 85–23, 1985. Animals were fed a normal protein diet (22% protein, Teklad No. 86 550; Teklad Premier Laboratory Diets, Madison, WI, USA). Glomerulonephritis was induced by tail vein injection of 2.25 mg/kg of the monoclonal anti-Thy 1.1 antibody OX-7 (NCCC; Biovest International, Inc., Minneapolis, MN, USA). OX-7 binds to a Thy 1-like epitope on the surface of mesangial cells, causing complement-dependent cell lysis followed by fibrotic tissue repair [36]. Control animals were injected with similar volumes of phosphate-buffered saline (PBS).

Table 1. Experimental design

Group	Treatment and (days of treatment)	Designation
1	PBS IV (0)	Normal control (NC)
2	OX7 IV (0), PBS IP (1, 3, and 5)	Disease control (DC)
3	OX7 IV (0), 1D11, 0.01 mg/kg IP (1, 3, and 5)	Ab 0.01
4	OX7 IV (0), 1D11, 0.1 mg/kg IP (1, 3, and 5)	Ab 0.1
5	OX7 IV (0), 1D11, 0.5 mg/kg IP (1, 3, and 5)	Ab 0.5
6	OX7 IV (0), 1D11, 5 mg/kg IP (1, 3, and 5)	Ab 5.0
7	OX7 IV (0), enalapril 200 mg/L in the drinking water (1–6)	Enal
8	OX7 IV (0), enalapril 200 mg/L in the drinking water (1–6), 1D11 0.5 mg/kg IP (1, 3, and 5)	Enal + Ab 0.5
9	OX7 IV (0), enalapril 200 mg/L in the drinking water, 1D11 5 mg/kg IP (1, 3, and 5)	Enal + Ab 5.0
10	OX7 IV (0), 13C4 (Control antibody), 5 mg/kg IP (1, 3, and 5)	Control Ab

Experimental design

A pilot experiment was carried out to determine the effective dose range of 1D11 in anti-Thy 1 glomerulonephritis. Doses of 1D11 from 0.05 to 5 mg/kg were administered to 10 groups of 4 rats. Based on the results, a larger experiment was carried out.

Groups of 8 rats were assigned and treated as outlined in Table 1. We have previously published that doses of enalapril above 100 mg/mL in drinking water are maximally effective in this model [13]. A dose of 200 mg/mL enalapril was used here. Enalapril treatment began 24 hours after disease induction. At that time, based on an average water intake of 40 mL per day, 60% of the daily dose was administered by gavage as described previously [13]. The pilot results indicated that the dose response was in the range 0.01 to 5 mg/kg; 1D11 doses of 0.5 and 5.0 mg/kg maximally reduced ECM accumulation, so these were used in combination with enalapril in groups 8 and 9.

Six days after induction of glomerulonephritis the animals were anesthetized with isoflurane. Following a midline abdominal incision, 5 to 10 mL blood was drawn from the lower abdominal aorta, and kidneys were subsequently perfused with 30 mL ice-cold PBS. For histologic examination cortical tissue was snap frozen or fixed in 10% neutral buffered formalin. Glomeruli from individual rats were isolated by a graded sieving technique (150, 125, and 75 μm mesh metal sieves) as described previously [15], and resuspended at 5000 glomeruli/mL/well in RPMI supplemented with 0.1 U/mL insulin, 100 U/mL penicillin, 100 μg/mL streptomycin, and 25 mmol/L

HEPES buffer. After a 48-hour incubation at 37°C/5% CO_2 the supernatant was harvested and stored at −70°C until analysis of glomerular production of fibronectin (FN), PAI-1, and TGF-β1.

Measurement of FN, PAI-1, and TGF-β1

FN and PAI-1 synthesis were measured with modified enzyme-linked immunosorbent assay (ELISA) according to published methods [37]. TGF-β1 production of cultured glomeruli was measured after acid-activation using a commercially available ELISA kit (DuoSet®; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Three samples from each rat were analyzed.

Light microscopy

All microscopic examinations were performed in a blinded fashion. Three μm sections of paraffin-embedded tissue were stained with periodic-acid Schiff (PAS), and glomerular matrix expansion was evaluated as previously described [15]. Briefly, in 30 glomeruli from each rat, the percentage of mesangial matrix occupying each glomerulus was rated as 0 = 0%, 1 = 25%, 2 = 50%, 3 = 75%, and 4 = 100%.

Immunofluorescent staining

Goat antihuman collagen I antibody was obtained from Southern Biotechnology Associates, Inc. (Birmingham, AL, USA). Fluorescein isothiocyanate (FITC)-conjugated rabbit antigoat immunoglobulin G (IgG; Dako Corporation, Carpinteria, CA, USA) was used as secondary antibody. Monoclonal mouse anticellular FN EDA^+ was obtained from Harlan Sera-Lab, Ltd. (Loughborough, England). FITC-rat $F(ab')_2$ antimouse IgG(H+L) was used as secondary antibody (Jackson Immunoresearch, West Grove, PA, USA). FITC-conjugated mouse antirat monocyte/macrophage antibody (ED1) was obtained from Serotec (Oxford, UK).

Renal tissue was snap frozen in 2-methylbutane that had been precooled at −80°C. Four-μm sections were obtained using a cryostat (Leica CM 1800; E Licht Company, Denver, CO, USA). Tissues were air dried and fixed in alcohol, washed in PBS, pH 7.4, incubated with the primary antibodies, washed with PBS, incubated with the appropriate FITC-conjugated secondary antibodies, washed again, and mounted with cover glasses using Fluoromount-G (Southern Biotechnology Associates, Inc.).

The intensity of glomerular staining of collagen I and FN was evaluated according to a 0 to 4 scale, which has been described in detail previously [38]. The number of cells per glomerulus staining positive for the monocyte/macrophage marker ED1 were counted. Thirty glomeruli per sample were evaluated. The mean values with standard error values were calculated.

RNA preparation and Northern hybridization

Total RNA was extracted by a guanidinium isothiocyanate method using Trizol® Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA from 8 rats of each group was pooled for further examination. For Northern analysis, RNA was denatured and fractionated by electrophoresis through a 1.0% agarose gel (30 μg/lane) and transferred to a nylon membrane (BrightStar™-Plus; Ambion, Inc., Austin, TX, USA). Nucleic acids were immobilized by ultraviolet irradiation (Stratagene, La Jolla, CA, USA). Membranes were prehybridized with ULTRAhyb™ buffer (Ambion, Inc.), and hybridized with DNA probes labeled with ^{32}P-dATP by random oligonucleotide priming (Strip-EZ DNA™; Ambion, Inc.). The blots were washed in 2 × standard saline citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) at room temperature for 10 minutes and in 0.1 × SSC, 0.1% SDS at 42°C for 15 minutes 2 times. DNA probes used were: (*1*) mouse 18S (ATCC, Manassas, VA, USA); (*2*) rat procollagen α_1 cDNA (kindly provided by Dr. D. Rowe) [39]; (*3*) FN-EDA cDNA (kindly provided by Dr. R.O. Hynes) [40]; (*4*) PAI-1 cDNA (kindly provided by Dr. T.D. Gelehrter) [41]; and (*5*) TGF-β1 cDNA (kindly provided by Dr. H. Moses) [42]. Three blots were performed for each probe. Autoradiographic signals obtained with 18S cDNA probe served as controls for equal loading of the gel. Autoradiographs were scanned on a Bio-Rad GS-700 imaging densitometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Changes in mRNA levels were determined by first correcting for the densitometric intensity of 18S for each sample. For comparison, this ratio was set at unity for normal control samples, and other lanes on the same gel were expressed as fold increases over this value.

Western blot

Glomeruli from individual rats were isolated and resuspended at 2×10^4 glomeruli/mL in radioimmunoprecipitation assay (RIPA) buffer (50 mmol/L Tris/HCl, pH 7.5, 150 mmol/L NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and 1 tablet/5 mL protease inhibitor mix [Complete, mini; Roche Diagnostics Corp., Indianapolis, IN, USA], 50 μL/5 mL phosphatase inhibitor cocktail II (Sigma). Glomeruli were homogenized 2 times on ice by sonication. Each 15-second sonication was followed by a 15-second cool down. After 2 centrifugations at 10,000*g* for 10 minutes at 4°C, the supernatant was stored at −70°C until analysis. Forty μL of each supernatant was separated by 10% Tris-glycine gel electrophoresis and transferred to a 0.45-μm nitrocellulose membrane (Millipore, Bedford, MA, USA). Nonspecific

binding was blocked by 10% nonfat milk powder in Tris-buffered saline (TBS) for 1 hour at room temperature, followed by 4°C overnight incubation with primary antibody [anti–phospho-Smad2; Upstate, Lake Placid, NY, USA, diluted 1:500 in 5% bovine serum albumin (BSA) in TBS/0.1% Tween-20 with 0.02% NaN_3; or anti–β-actin, diluted 1:10,000 (Sigma)]. The blot was washed 3 times for 10 minutes in TBS/0.1% Tween-20. The second antibody, goat antirabbit horseradish peroxidase or goat antimouse horseradish peroxidase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), was incubated at a dilution of 1:2000 for an additional hour at room temperature, followed by 3 washes as described above. Bound antibodies were detected by developing the blot in enhanced chemiluminescence (ECL)™ Western blotting detection reagents (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) for 1 minute. Quantitation of the bands on autoradiograms was performed using a Bio-Rad GS-700 imaging densitometer. Changes in p-Smad2 levels were determined by correcting for the densitometric intensity of β-actin for each sample.

Statistical analysis and calculation of percent reduction in disease severity

Data are expressed as mean ± SEM. Statistical analysis of differences between the groups was performed either by analysis of variance (ANOVA) and subsequent Student-Newman–Keuls or Dunnett testing for multiple comparison, or, in the case of PAS scoring, collagen I and FN immunofluoresence staining, differences between the groups were tested using analysis of ranks by Kruskal-Wallis with Dunn's post analysis. Mean or median values were considered significantly different when $P < 0.05$.

The disease-induced increase in a variable was defined as the mean value for the disease control group minus the mean value of the normal control group. The percent reduction in disease severity in a treated group was calculated as:

$$\frac{\text{Disease control group mean} - \text{Treated control group mean}}{\text{Disease control group mean} - \text{Normal control group mean}} \times 100$$

RESULTS

Three questions were asked in these experiments. First, is 1D11 effective in this model of nephritis and can we determine the maximally effective dose of 1D11? Second, how does this dose compare with effects seen with maximal doses of enalapril? And third, can we see additivity of enalapril and 1D11 when both are administered at maximally effective doses?

Effect of 1D11

The induction of disease by the administration of OX-7 produced a rapid accumulation of extracellular matrix in glomeruli as measured by PAS staining. Representative glomeruli from this study are shown in Figure 1. The disease-induced increase in ECM is seen when Figure 1A (normal control) and Figure 1B (disease control) are compared. Increasing doses of 1D11 (0.05 mg/kg to 5.0 mg/kg), shown in Figure 1C to F, reveal a clear decrease in PAS-positive material with increasing doses of 1D11. To quantify this histologic effect of 1D11 on the accumulation of ECM, 30 glomeruli per animal were scored for PAS staining, scores were averaged for each animal, and then for each group. This analysis, presented graphically in Figure 2A, indicates that 1D11 produced a dose-dependent reduction in PAS staining. A moderate but significant ($P < 0.05$) decrease of approximately 56% was seen for PAS staining in rats treated with 0.5 mg/kg 1D11 compared to disease control animals.

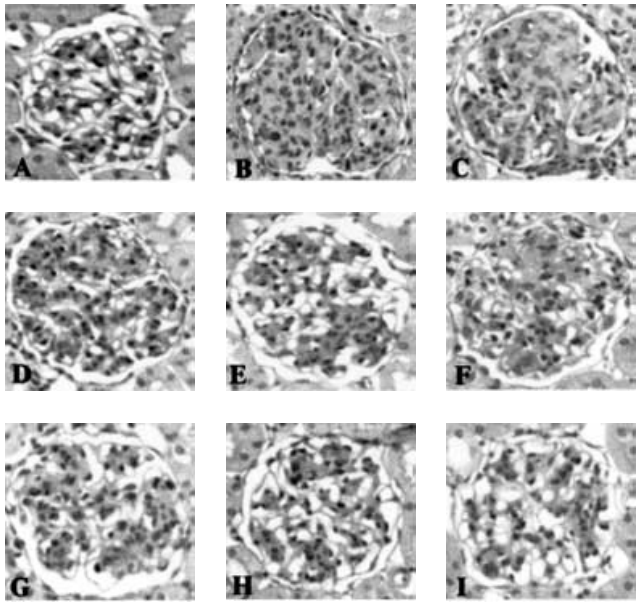


Fig. 1. Effect of increasing doses of 1D11, enalapril, and combinations of 1D11 and enalapril on the development of glomerulosclerosis. Paraffin sections. Magnification, × 400. Compared with normal rats (*A*), glomerular enlargement and significant mesangial matrix deposition were evident in untreated, disease control (*B*). These changes were ameliorated by doses of 1D11 0.01 (*C*), 0.1 (*D*), 0.5 (*E*), 5.0 (*F*) mg/kg, and enalapril (*G*). The best therapeutic effects were achieved by combination therapies Enal+0.5 (*H*) and Enal+5 (*I*).

In order to determine the contribution of specific matrix proteins to the PAS-positive material immunofluorescent staining for specific glomerular proteins was performed. Representative glomeruli stained for FN EDA^+ and type I collagen are shown in Figures 3 and 4. Again, a dramatic increase in staining for matrix proteins was seen in disease control animals (Figs. 3B and 4B) (up to 13-fold for collagen and 3.6-fold for FN) compared to normal control animals (Figs. 3A and 4A). Treatment with 1D11 produced dose-dependent decreases in the accumulation of FN EDA^+ and collagen I in glomeruli which are clearly seen when disease control glomeruli (Figs. 3B and 4B) are compared with increasing doses

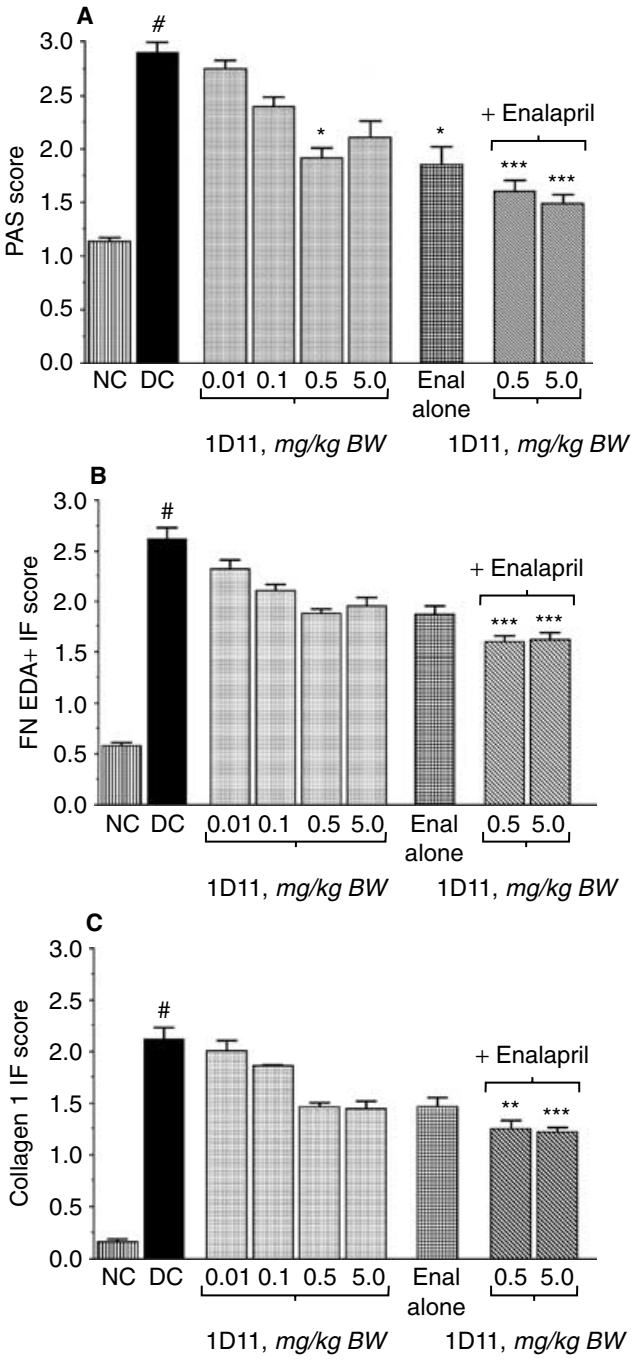


Fig. 2. Effect of increasing doses of 1D11, enalapril, and combinations of 1D11 and enalapril on matrix score (*A*), and immunohistochemical staining for fibronectin EDA+ (*B*) and collagen I (*C*). #$P < 0.001$ disease control (DC) compared to normal control (NC) group; *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ treatment groups compared to DC group.

of 1D11 as shown in Figures 3C to F and 4C to F. As with PAS scores, all staining scores were averaged and are presented graphically in Figures 2B and 2C. Doses of 0.5 mg/kg and 5 mg/kg 1D11 reduced both FN EDA$^+$ and collagen I deposition between 32% and 36%, respectively; however, these changes were not significant.

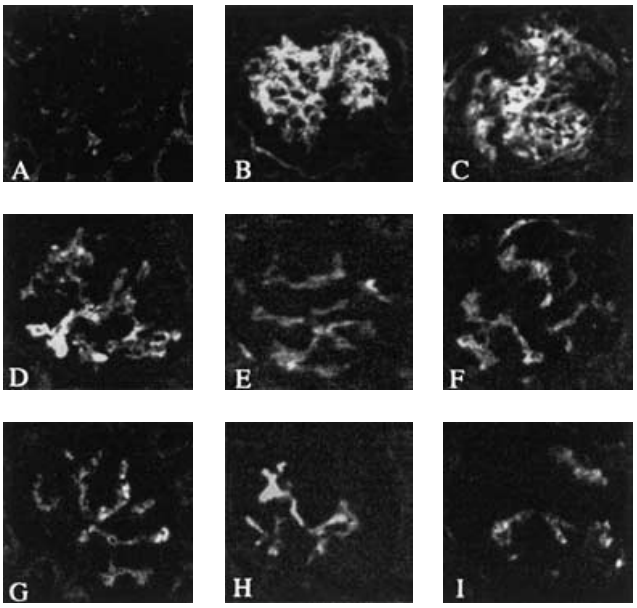


Fig. 3. Immunofluorescence micrographs of fibronectin EDA$^+$. Magnification, × 400. A weak immunoreactivity was observed in normal glomeruli (*A*). Remarkably strong intraglomerular staining of fibronectin was found in untreated rats (*B*). Fibronectin accumulation was ameliorated by 1D11 treatment 0.01 (*C*), 0.1 (*D*), 0.5 (*E*), 5 (*F*) mg/kg, and enalapril (*G*). The best therapeutic effects were achieved by combination therapies Enal+0.5 (*H*) and Enal+5 (*I*).

Glomerular synthesis of proteins of interest was determined by ELISA on culture supernatants from isolated glomeruli cultured for 48 hours. Again, as shown in Figure 5, 1D11 treatment caused a dose-related reduction of these markers of disease. At a 1D11 dose of 5 mg/kg, FN, PAI-1, and TGF-β1 levels were reduced by 54%, 115%, and 67%, respectively ($P < 0.05$).

Glomerular mRNA expression of FN EDA$^+$, collagen I, PAI-1, and TGF-β1 is shown in Figures 6 and 7. Blots for one gel are shown in Figure 6. The mean ± SE of densitometry values obtained from scans of 3 gels are shown graphically in Figure 7. 1D11 treatment reduced gene expression of FN EDA$^+$ and PAI-1, collagen I, and TGF-β1 mRNA in a dose-dependent manner. However, the maximal effect for the inhibition of FN EDA$^+$, collagen I, and TGF-β1 expression was achieved at a dose of 0.5 mg/kg, with 5 mg/kg appearing to be less effective. 1D11 maximally reduced FN EDA$^+$ expression by 63%, PAI-1 by 58%, collagen I by 96%, and TGF-β1 by 48% (Fig. 7A to D, respectively).

Treatment with 1D11 also reduced proteinuria in a dose-dependent manner. 1D11 at 5 mg/kg maximally reduced proteinuria, giving mean urinary proteins of 17.7 ± 2.3 mg/24h, compared to the disease control group (35.1 ± 8.5 mg/24h). However, for this readout, the control antibody 13C4 mouse IgG1 (5 mg/kg) also reduced proteinuria to 10.2 ± 0.9mg/24h. Hence, it is difficult to interpret the effect of 1D11 on this variable. The control

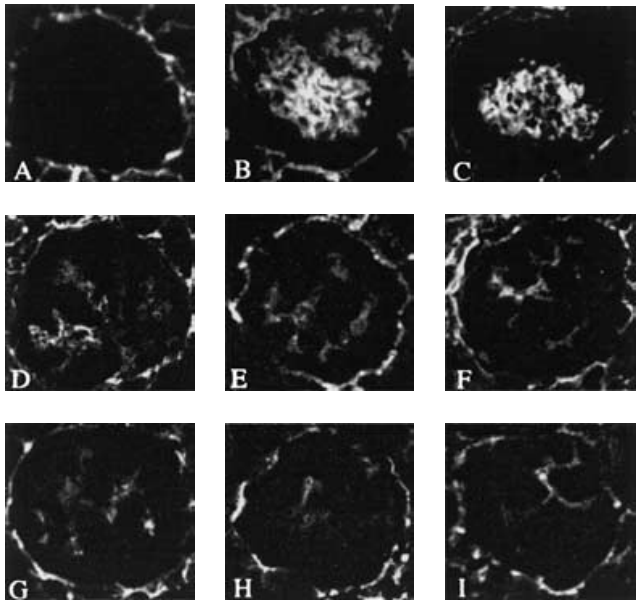


Fig. 4. Immunofluorescence micrographs of type I collagen. Magnification, × 400. Tubular basement membrane and Bowman's capsule were positively stained with collagen I antibody. Nearly no immunoreactivity was seen in normal glomeruli (*A*). Remarkably strong intraglomerular staining of collagen I was found in untreated disease control rats (*B*). Collagen I accumulation was ameliorated to different extents by 1D11 treatments 0.01 (*C*), 0.1 (*D*), 0.5 (*E*), 5 mg/kg (*F*), and enalapril (*G*). The best therapeutic effects were achieved by combination therapies Enal+0.5 (*H*) and Enal+5 (*I*).

antibody, 13C4, had no significant effect on any other measure of disease (compared to the disease control, data not shown).

Comparison of the effect of 1D11 and enalapril

From data presented above, it is clear that either 0.5 or 5.0 mg/kg 1D11 show maximal therapeutic effects in this model. Also, from our previous dose-response study with enalapril [13], we know that enalapril, at doses greater than 100 mg/L in drinking water, produces the maximally obtainable therapeutic response in this model. Thus, 200 mg/L enalapril alone or in combination with 0.5 or 5 mg/kg 1D11 was used to determine the effect of combining these 2 treatments.

The 0.5 mg/kg dose of 1D11 reduced matrix score by 56%, whereas enalapril alone reduced it by 59% (Figs. 1E, G, and 2A). This similarity in disease reduction was seen for most measures of disease. Enalapril or maximally effective 1D11 (either 0.5 mg/kg or 5 mg/kg) reduced FN immunostaining by 36% and 36% (Figs. 2B, 3E, and G), collagen I immunostaining by 33% and 34% (Figs. 2C, 4E, and G), and FN production by 53% and 54%, respectively (Fig. 5A).

In contrast, the therapeutic effect of 1D11 on PAI-1 production by cultured glomeruli (Fig. 5B) and on PAI-1 mRNA (Figs. 6 and 7B) appears to be somewhat

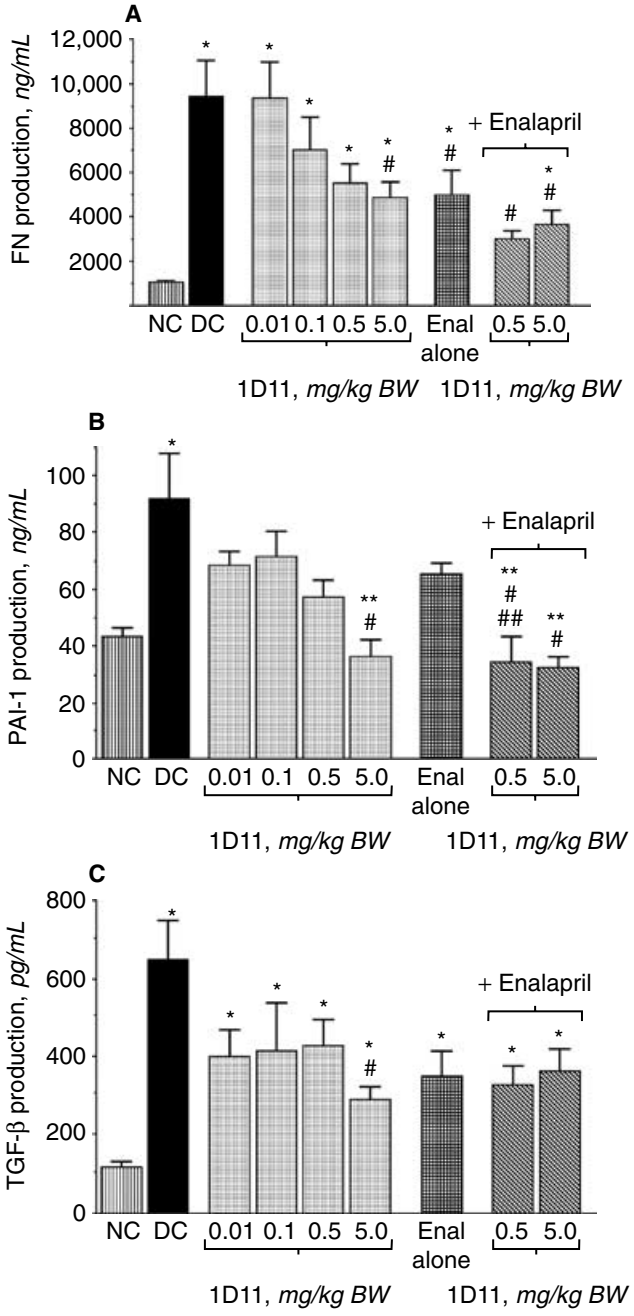


Fig. 5. Effect of increasing doses of 1D11, enalapril, and combinations of 1D11 and enalapril on glomerular production of fibronectin (*A*), PAI-1 (*B*), and TGF-β1 (*C*). $^{*}P < 0.05$ compared to normal control (NC) group; $\#P < 0.05$ compared to disease control (DC) group; $^{**}P < 0.05$ compared to enalapril alone; $\#\#P < 0.05$ compared to the corresponding dose of antibody alone.

greater than that seen for enalapril, although statistical tests did not reach significance for comparisons between these groups. Similarly, the mRNA analysis suggested that 1D11 was much more effective in reducing collagen I mRNA than was enalapril ($P < 0.05$; Figs. 6 and 7C).

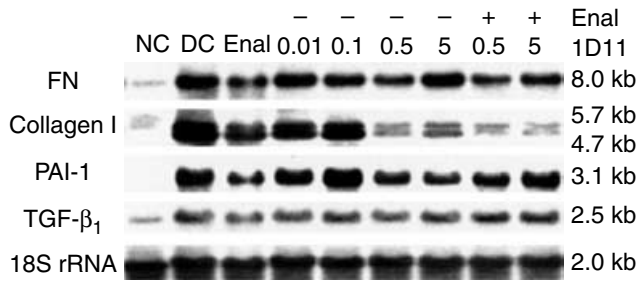


Fig. 6. A representative Northern blot showing glomerular mRNA expression of TGF-β1, PAI-1, collagen I, and fibronectin harvested six days after induction of glomerulonephritis.

Combination of 1D11 and enalapril

It is clear from the data that combining maximally effective doses of enalapril and 1D11 confers additional therapeutic benefits compared to either drug given alone. Combination of enalapril with 1D11 reduced glomerular matrix accumulation, as measured by PAS staining, from approximately 59% enalapril alone or 1D11 alone to 80% for enalapril plus 5.0 mg/kg 1D11 ($P < 0.001$ compared to disease control animals) (Figs. 1E to I and 2A). The enhanced therapeutic effect of combined treatment with both agents was also seen for FN (Figs. 2B and 3) and collagen I (Figs. 2C and 4) immunostaining, as well as glomerular production of FN and PAI-1 (Fig. 5A and B). Addition of 1D11 (0.5 mg/kg) to enalapril further reduced FN production by 25% to a total inhibition of 77%. In terms of proteinuria, monotherapy reduced disease-induced increases in urinary protein by 78%, 74%, and 62% for enalapril, 0.5 1D11, and 5 1D11, respectively. Combination treatment further reduced proteinuria to 98% and 99% for 0.5 and 5.0 mg/kg 1D11, respectively, values that did not differ from normal. Combination treatment had no further effect on PAI-1 production. However, this variable was reduced to levels lower than those of the normal control group, as with 1D11 alone (Fig. 5B). In contrast, as shown in Figure 5C, the treatment-related reduction in TGF-β1 production by cultured glomeruli was similar across all treatment groups, indicating no additive effects of combination on this readout.

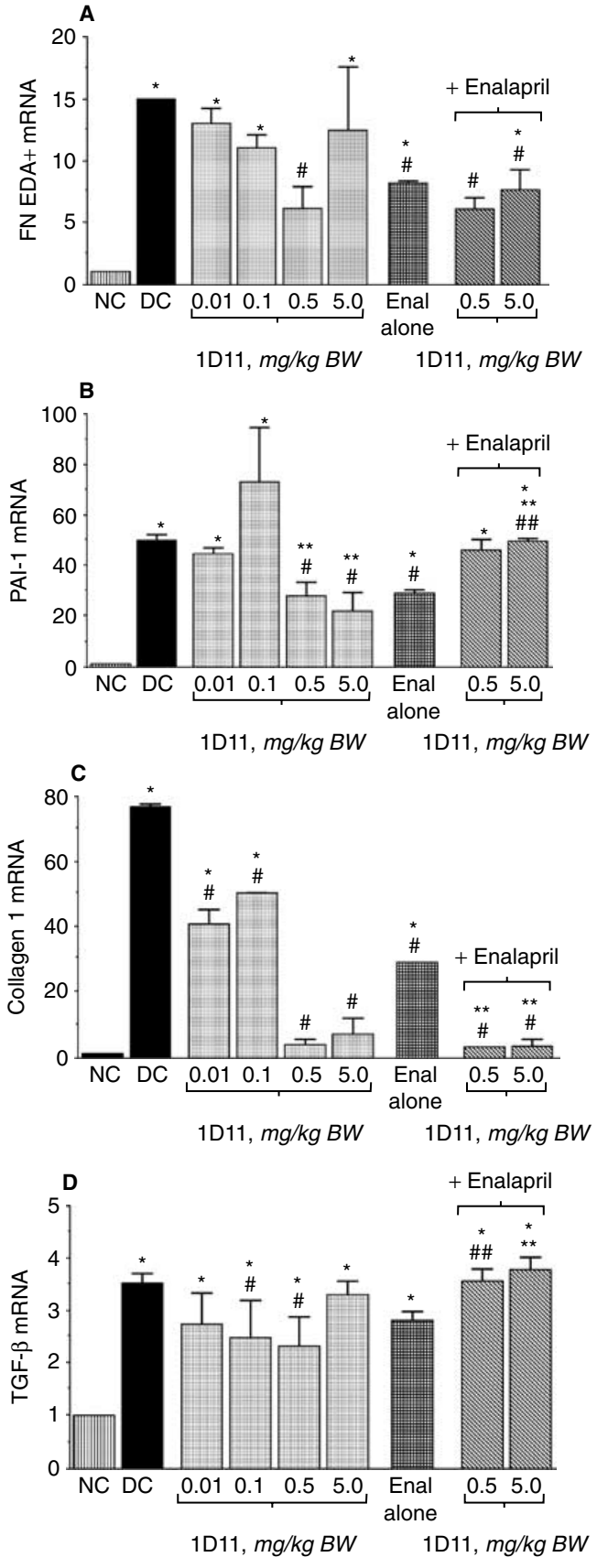
The results obtained for glomerular mRNA levels for FN, collagen I, TGF-β1, and PAI-1 produced a more mixed picture than other variables, and one that did not entirely agree with the results of protein production by cultured glomeruli (Figs. 6 and 7). FN mRNA levels were similarly reduced whether enalapril or 1D11 were used alone and the maximum inhibition of FN mRNA was achieved with 0.5 mg/kg 1D11, with 5 mg/kg being less effective. However, the combination of enalapril with 1D11 5 mg/kg appeared to reverse this apparent reduction in efficacy of 1D11 restoring the inhibition of FN mRNA production (Fig. 7A). Interestingly, while the therapeutic effect of enalapril or 1D11 alone resulted in very similar reductions in PAI-1 mRNA, combination of these 2 drugs appeared to have reversed the effect for PAI-1 message (Fig. 7B). Compared to the protein levels measured in the supernatant, the data suggest that the increase in PAI-1 mRNA was not translated into PAI-1 protein production and secretion into culture supernatant. Collagen I mRNA was reduced to control levels by 1D11 treatment alone at both 0.5 and 5 mg/kg; therefore, it is not surprising that combination of 1D11 and enalapril had no further effect (Fig. 7C). Finally, the picture for TGF-β1 mRNA levels is also somewhat mixed (Fig. 7D). While enalapril or 0.5 mg/kg 1D11 reduced TGF-β1 mRNA by 29% and 48%, respectively, high-dose antibody or combinations entirely reversed this effect, bringing levels essentially to those of the disease control group (Fig. 7D). As with PAI-1 mRNA, this finding was seen only for TGF-β1 mRNA, and not for TGF-β1 secreted into culture supernatant by glomeruli. The significance of these mRNA data is therefore not fully supportive of the observed protein changes. The reason for this is unknown.

Studies into possible mechanisms of the additivity of enalapril and 1D11. Infiltration of macrophages into injured glomeruli is thought to contribute to disease in anti-Thy1 nephritis [43]. The results of staining for $ED1^+$ cells of the monocyte/macrophage lineage are shown in Figure 8. At the 3 low doses of antibody a clear dose-response is seen, with the number of $ED1^+$ cells being significantly ($P < 0.05$) reduced by 71% with the 0.5 mg/kg dose of 1D11. However, at the highest dose of 1D11, 5 mg/kg was less effective, such that the reduction was only 23% (Fig. 8). Enalapril alone also significantly ($P < 0.05$) reduced ED1+ cells by 62%. Interestingly, as with FN mRNA, combination of both drugs appeared to prevent the rebound seen with high-dose 1D11 and the reduction in $ED1^+$ cells was sustained at 70% and 69% in the two combination groups (Fig. 8).

To study intracellular TGF-β signal transduction, we analyzed activation of Smad2 using an antibody specifically detecting phosphorylated Smad2 (Fig. 9). Diseased glomeruli have dramatically increased intracellular p-Smad2 compared to normal control. 1D11 dose-dependently reduced Smad2 activation, with the maximal reduction of 75% at the highest dose. Enalapril also reduced Smad2 activation by 36%. Adding enalapril to 1D11 resulted in a further reduction. 5 mg/kg 1D11 plus enalapril nearly normalized p-Smad2 levels.

DISCUSSION

The data presented here show that increasing doses of the TGF-β1, -β2, and -β3 neutralizing antibody 1D11 results in increasing therapeutic efficacy. In this model of acute renal fibrosis, a dose-dependent reduction of fibrotic disease is observed until the maximal effect at



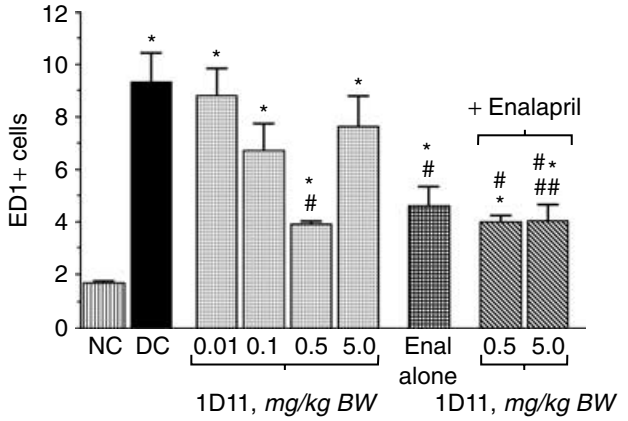


Fig. 8. Effect of increasing doses of 1D11, enalapril, and combinations of 1D11 and enalapril on immunofluorescent staining for the monocyte/macrophage marker ED1. $^{*}P < 0.05$ compared to normal control (NC) group; $\#P < 0.05$ compared to disease control (DC) group; $\#\#P < 0.05$ compared to the corresponding dose of antibody alone.

0.5 mg/kg 1D11, after which efficacy appears to plateau. Since some of the measures we employed to assess efficacy were actually less effectively blocked in the group receiving 5.0 mg/kg 1D11, it is likely that the true maximally therapeutic dose is between 0.5 and 5.0 mg/kg. The data suggest that the maximal disease reduction possible with 1D11 is close to 50%, and that the disease reduction of 1D11 given alone is comparable to enalapril given alone.

We have shown that a combination of TGF-β inhibition with Ang II blockade resulted in an enhanced disease reduction. Thus, the combination of Ang II blockade and TGF-β inhibition could represent a major step forward in efforts to halt disease in humans. While we have previously shown additivity in anti-Thy1 nephritis with L-arginine supplementation and low protein diet, and also with angiotensin blockade and low protein diet [44, 45], both the degree of additivity and the feasibility of the combination of Ang II blockade and TGF-β inhibition are clearly superior to the previously used combinations.

A series of studies in the anti-Thy 1 model have shown that monotherapy with enalapril, losartan, 1% L-arginine in drinking water, or 6% low protein diet all reduced markers of fibrotic disease about 50% [11, 43]. In the present study we add the neutralizing antibody to TGF-β1, -β2, and -β3, 1D11, to this list. This is a particularly

Fig. 7. Effect of increasing doses of 1D11, enalapril, and combinations of 1D11 and enalapril on glomerular mRNA levels for fibronectin (*A*), PAI-1 (*B*), collagen I (*C*), and TGF-β1 (*D*). Data presented are the means of densitometric scans of three Northern blots using pooled mRNA from each group of rats. $^{*}P < 0.05$ compared to normal control (NC) group; $\#P < 0.05$ compared to disease control (DC) group; $^{**}P < 0.05$ compared to enalapril alone; $\#\#P < 0.05$ compared to the corresponding dose of antibody alone.

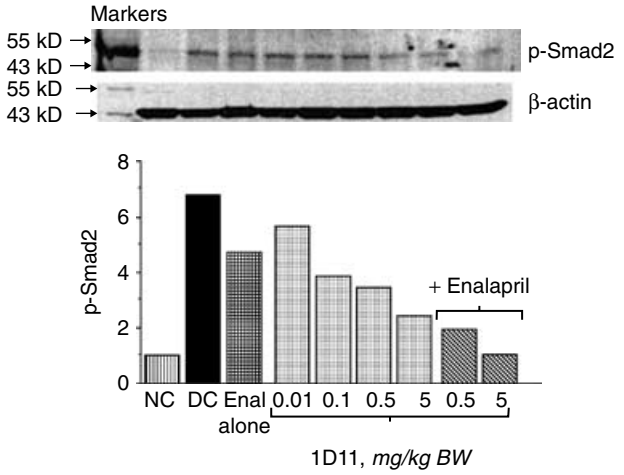


Fig. 9. Effect of increasing doses of 1D11, enalapril, and combinations of 1D11 and enalapril on glomerular p-Smad2 protein as determined by Western blotting.

important addition because, of the many molecules involved in fibrosis, none has been so consistently or thoroughly implicated as over-expression of TGF-β. Here, even with the potent anti–TGF-β neutralizing antibody 1D11, we see only a maximum 50% reduction in disease severity. The fact that no single treatment is able to prevent fibrotic disease suggests that either different pathways are mediating disease, or that the therapy itself stimulates back-up mechanisms to operate. Interestingly, the severity of fibrosis in the rat model of unilateral ureteral obstruction was also decreased about 50% in animals where both angiotensinogen genes were disrupted [46]. On the other hand, treatment with a TGF-β–neutralizing antibody (not 1D11) in a type II diabetic mouse model (*db/db*) for 8 weeks reduced glomerulosclerosis by 82% compared to normal littermates, suggesting that the maximal effectiveness of antibodies may differ with disease model [27].

It should be noted that a number of studies have shown additivity of suboptimal doses of various treatments including ACEI and AT_1RA [45, 47–50]. While combinations of suboptimal doses of two drugs may be clinically useful to reduce side effects of high doses of one drug, true additivity, or synergism, can only be shown when maximally effective doses of at least 1 of 2 drugs are given. This is the first demonstration of true additivity of ACE inhibition and TGF-β antibodies. The ability of 1D11 and enalapril to show additivity when given at maximally therapeutic doses suggests that these drugs act, at least in part, through different pathways. This is in contrast to results when enalapril and losartan were combined, and no additivity was seen [13], suggesting that these drugs work though a common pathway.

Inconsistent results in studies such as this are seen with respect to reductions in either TGF-β mRNA and/or TGF-β protein. While it might be expected that an association between disease severity and TGF-β expression would be seen, there are a number of studies where this was not seen. Here, we did not see a reduction in TGF-β mRNA with enalapril treatment, although we did in an earlier study [13]. Similarly, Benigni et al [50], using lisinopril and 1D11, did not see significant reductions in glomerular mRNA. Other studies with Ang II blockade and/or TGF-β antibody therapy have seen significant reductions in TGF-β mRNA [26, 30, 51]. One explanation for these inconsistencies is the necessity of pooling samples to obtain adequate RNA for Northern blots, resulting in reduced power to detect differences. It is clear, however, that whether TGF-β over-expression is reduced or not, expression of downstream fibrogenic molecules, such as collagen I and FN, is generally significantly reduced.

To help clarify the mixed picture of TGF-β expression in this and other studies, we looked further into TGF-β signal transduction system. Using p-Smad2 as an activation marker, we found 1D11 dose-dependently reduced TGF-β signal transduction, and so did maximally effective doses of enalapril. Combination therapy resulted in further reduction of p-Smad2 compared to monotherapy. This result is consistent with the reductions seen for other measures of disease severity. It is probable that the enalapril effect on Smad activation is due to blocking Ang II–induced up-regulation of TGF-β or TGF-β receptor expression [52–55].

An interesting finding in the study presented here is that the 1D11 dose response is modulated by the addition of enalapril. Reversal of the therapeutic effect of 1D11 on matrix score (Fig. 2A), FN mRNA (Fig. 7A), collagen I mRNA (Fig. 7C), and TGF-β1 mRNA (Fig. 7D) was seen at the highest dose of 1D11 (5.0 mg/kg). While this reversal was often marginal for all but FN, the results do hint that more complete inhibition of TGF-β may be less effective at treating disease. This is most clearly seen in Figure 8, where the highest dose of 1D11, 5 mg/kg, is less effective at reducing $ED1^+$ cell influx than a lower dose of this agent. Interestingly, a similar effect on monocyte/macrophage cells with the 5.0 mg/kg dose of 1D11 was recently reported in the puromycin nephropathy model [abstract; Ma LJ et al, *J Am Soc Nephrol* 12:2001:819A]. In the present study, the therapeutic effect was also reduced with this dose of 1D11. Both Ang II and TGF-β play important roles in recruitment of activated macrophages to the site of injury. These activated macrophages are thought to release chemotactic and profibrotic factors. TGF-β, however, has dual effects on macrophages. It strongly attracts macrophages and at the same time inhibits activated macrophage function by reducing the production and release of other chemotactic and inflammatory factors [56–58]. It is possible that increasing doses of 1D11 decrease macrophage infiltration initially, but as more TGF-β is neutralized,

escape from TGF-β inhibition may lead to release of agents that further recruit macrophages. While the reduction of effect at higher doses of 1D11 and the possible role of macrophages in this observation requires further study, it is very interesting that combination of high-dose 1D11 with enalapril appears to reverse this tendency to rebound for all variables, except for TGF-β1 mRNA. This supports the notion that combination of Ang II blockade with TGF-β antibodies will not only provide greater therapeutic benefit than currently available therapies, but that any potential negative consequences may be ameliorated by this drug combination.

Finally, it is noteworthy that TGF-β antibody treatment did not cause any noticeable side effects. This is not surprising since this short-term experiment lasted only 6 days. Other long-term studies with TGF-β antibody, including 4 publications with the mouse monoclonal antibody, 1D11 used here [27, 28, 30, 50, 51, 59], reported no safety problems with this therapy.

CONCLUSION

The study presented here demonstrates a clear dose-dependent therapeutic response for the TGF-β1, -β2, and -β3 neutralizing antibody, 1D11. The maximal therapeutic effect of monotherapy is close to 50% disease reduction, whether the therapy is Ang II blockade or TGF-β inhibition. Finally, and most importantly, the data show an additional reduction in disease severity when maximally effective doses of enalapril are combined with TGF-β antibody, a finding that may stimulate human trials of such combinations and eventually better therapy for human fibrotic kidney disease.

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Reprint requests to Nancy A. Noble, Ph.D., Fibrosis Research Laboratory, 391 Chipeta Way, Suite E, Salt Lake City, UT 84108.
E-mail: nancy.noble@hsc.utah.edu

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