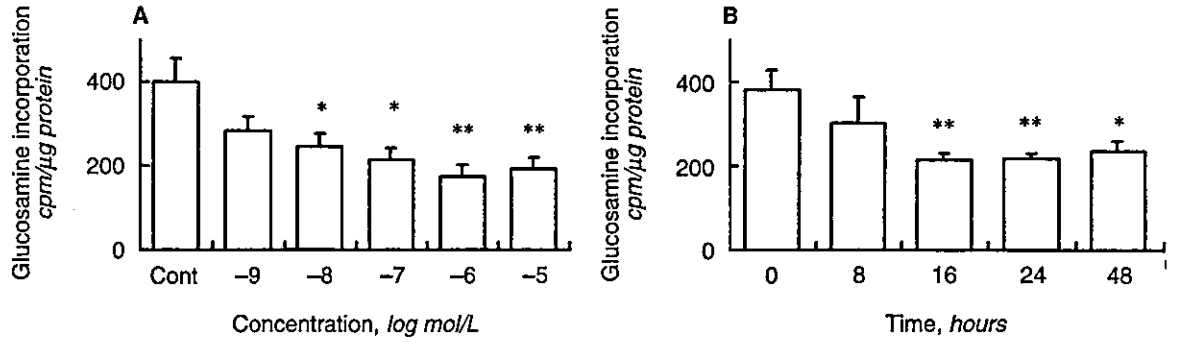

Fig. 2. (A) Dose dependency and (B) time course of dexamethasone-induced changes in proteoglycan synthesis in RMC. Quiescent RMC were treated with (A) various doses of dexamethasone for 48 hours or (B) with 1 μmol/L dexamethasone for various times, then proteoglycan synthesis in the media was assayed as described in the **Methods** section. In panel B the total labeling time was 48 hours. Dexamethasone was added at the indicated times before the end of the labeling period. Results shown are the mean ± SEM ($N = 4$ per assay point). *$P < 0.05$, **$P < 0.01$ vs. control.

Table 1. Analysis of proteoglycan subtype synthesis in media from rat mesangial cells (RMC) with and without dexamethasone treatment

	Incorporation *cpm/μg protein*			
	Dex (−)		Dex (+)	
	^{3}H-glucosamine	^{35}S-sulfate	^{3}H-glucosamine	^{35}S-sulfate
Chondroitinase ABC-sensitive incorporation	126 ± 14	320 ± 15	109 ± 8	254 ± 27*
Chondroitinase AC-sensitive incorporation	78 ± 10	218 ± 12	47 ± 11*	170 ± 12*
Heparitinase-sensitive incorporation	23 ± 8	63 ± 16	18 ± 6	55 ± 24

Results shown are the mean + SEM ($N = 6$).
*$P < 0.05$

tinase, or undigested were 58%, 36%, 11%, 31% in the control cells, and 56%, 24%, 9%, 35% in the dexamethasone-treated cells, respectively). The changes in the chondroitinase ABC-, AC- and heparitinase-sensitive counts with and without dexamethasone treatment are shown in Table 1. Treatment of cells with dexamethasone appeared to cause reductions predominantly in chondroitinase ABC- and AC-sensitive proteoglycans (Table 1). To confirm and clarify these findings, characterization of the proteoglycan subclass was also performed by ion-exchange chromatography using DEAE-Sephacel (Fig. 3). In the supernatants from both control and dexamethasone-treated samples, incorporated ^{35}S-sulfate radioactivity eluted from the ion exchange column predominantly at two peaks. The counts from peak I and II were attenuated by pretreatment with heparitinase and chondroitinase ABC, respectively, as reported previously [22], suggesting that these peaks contained predominantly HSPG and CS/DSPG. A clear reduction in peak II was seen in the dexamethasone-treated samples, whereas no major changes in peak I were observed, suggesting a reduction in CS/DSPG consistent with the results of the enzyme digestion experiments. Next, the DEAE-Sephacel peak II samples were applied to a Sepharose CL-2B molecular sieve column. As shown in Figure 4, no differences in the elution position of proteoglycans from the

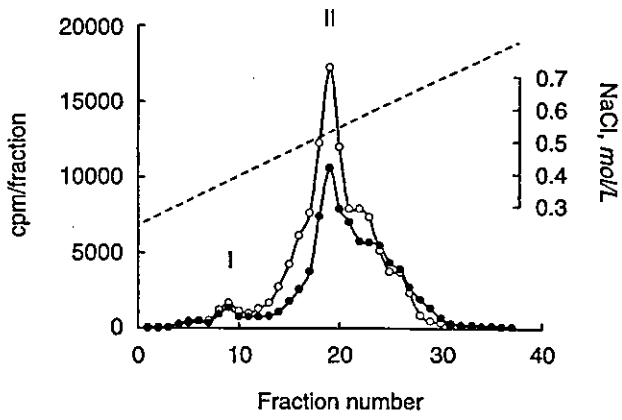

Fig. 3. Analysis of proteoglycans in media from control (○) and dexamethasone-treated (●) RMC by DEAE-Sephacel ion-exchange chromatography.

control and dexamethasone-treated cells were observed, suggesting that dexamethasone did not cause a major change in the length of the proteoglycan molecules.

Effects of dexamethasone on proteoglycan core protein mRNA and protein

To examine if the changes in proteoglycan synthesis mediated by dexamethasone involved changes in proteo-

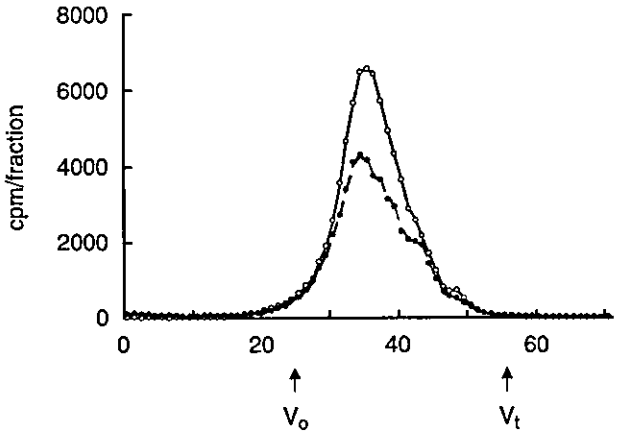

Fig. 4. Analysis of proteoglycans in media from control (○) and dexamethasone-treated (●) RMC by Sepharose CL-2B gel permeation chromatography. Samples were applied after initial fractionation by ion-exchange chromatography, as described in the **Methods** section. Abbreviations are: V_o, void volume; V_t, total volume.

glycan core protein mRNA, Northern blot analysis was performed to assess changes in the major proteoglycan core proteins expressed in mesangial cells. Since the signals obtained by Northern blot were low in some cases, RT-PCR also was performed as described previously by our group. Comparison of the data obtained using these two assay methods revealed consistent results as shown in Figure 5. Treatment of RMC with dexamethasone caused a significant ($P < 0.05$) decrease in decorin mRNA, while a similar but non-significant trend was seen for versican. On the other hand, expression of biglycan mRNA was unexpectedly increased by the dexamethasone treatment. To confirm these findings, expression of the proteoglycan core proteins decorin and biglycan in control and dexamethasone-treated samples were examined by Western blot analysis. These experiments were performed using HMC, since the antibodies used (LF-136 and LF-51) were raised against the human core proteins. Bands of the expected size (approximately 45 kD) were visible after chondroitinase ABC digestion. Consistent with the results of Northern blot analysis and RT-PCR, expression of decorin appeared decreased, whereas biglycan protein appeared increased in the dexamethasone-treated samples compared to the controls, suggesting differential regulation of these two proteoglycans (Fig. 5E).

Effects of dexamethasone on biglycan promoter activity

To examine the mechanisms of the dexamethasone-induced increase in biglycan mRNA, RMC were transfected with biglycan promoter constructs using lipofectamine, and promoter activity with or without dexamethasone treatment was assessed by the dual luciferase assay. As shown in Figure 6, dexamethasone caused a significant increase (2- to 3-fold) in the promoter activity of the biglycan construct Bgn (−1212, +42), as well as the shorter constructs Bgn (−985, +42) and Bgn (−686, +42). In contrast, no significant increase in promoter activity was induced by dexamethasone in the case of the truncated constructs Bgn (−152, +42) and Bgn (−46, +42). Of interest, these last two truncated constructs lacked the two putative GRE motifs in the biglycan promoter region.

Effects of in vivo dexamethasone treatment on decorin and biglycan mRNA in isolated glomeruli

To confirm that the dexamethasone-induced changes in decorin and biglycan mRNA were relevant to the in vivo situation, SD rats were treated with dexamethasone in vivo, then expression of decorin and biglycan mRNA was examined by RT-PCR. As shown in Figure 7, treatment of rats for three days with dexamethasone (5 mg/kg/day) caused a significant increase in biglycan mRNA, in contrast to decorin, which showed a tendency to decrease after steroid treatment, consistent with the in vitro results.

DISCUSSION

The extracellular matrix is known to be a rich source of growth factors, cytokines, and signaling molecules that interact with each other and together regulate cellular function. A key component of this matrix is the biochemically distinct group of glycoproteins known as proteoglycans, which consist of a core protein covalently linked to one or more GAG side chains [1]. These proteoglycans have multiple functions, including the control of extracellular matrix assembly through interactions with collagen proteins, the activation and inactivation of growth factors that may be directly responsible for the development and progression of glomerular disease, as well as direct effects on cellular actions by interacting with cell surface receptors and adhesion molecules.

Recent studies have shown that the expression of proteoglycans changes markedly during the course of renal disease, and that these changes may play an important role in the pathogenesis of various common glomerular diseases. In primary glomerulonephritis, up-regulation of the CS/DSPGs biglycan and decorin is seen, and the temporal and spatial patterns of expression of these proteoglycans are consistent with a role for these proteoglycans in the pathogenesis of rapidly progressive glomerulonephritis [4, 5, 23]. On the other hand, a decrease in glomerular HSPGs has been linked to the proteinuria seen in the nephrotic syndrome. A similar increase in glomerular biglycan and decorin production together with a decrease in HSPG has been found in diabetic nephropathy [6–8]. Urinary excretion of decorin has been found to be increased in both membranous nephropathy and diabetic

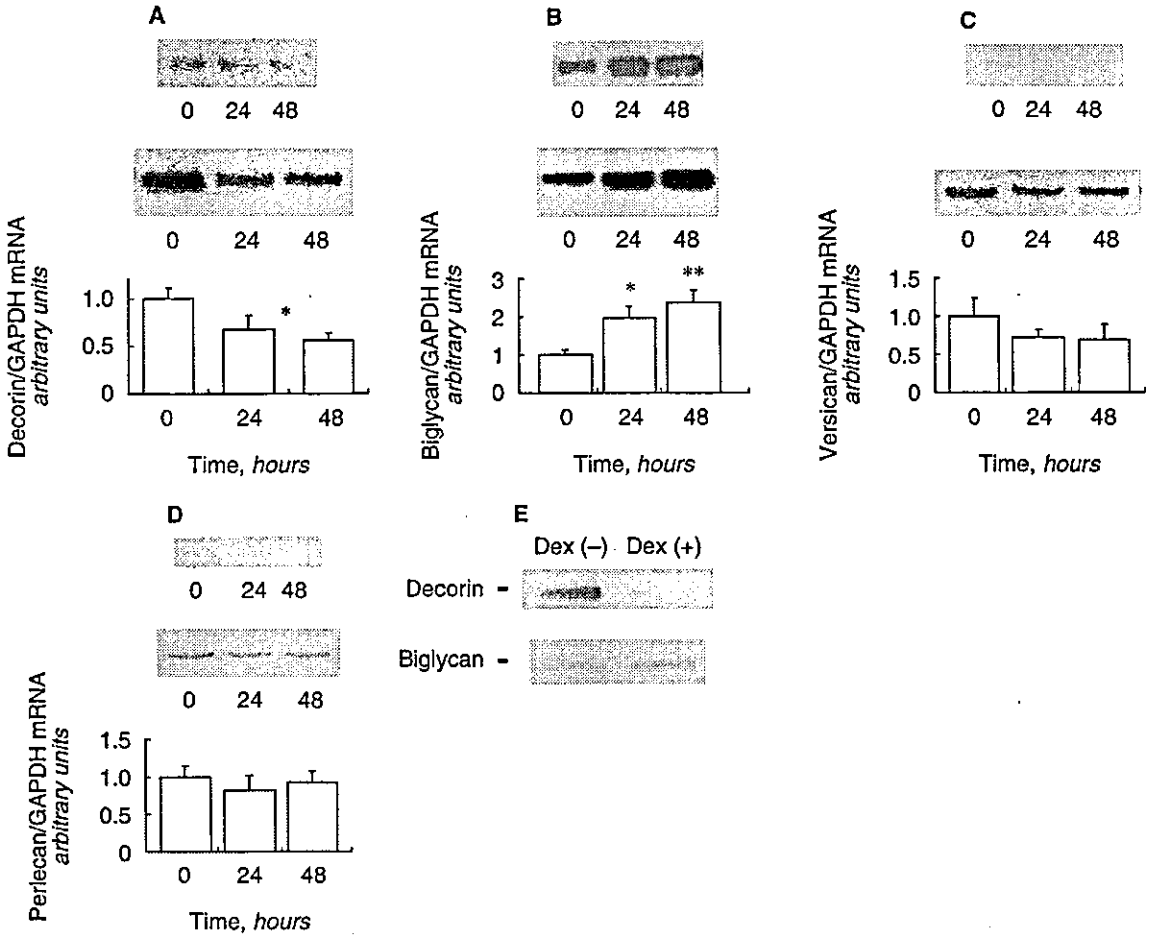

Fig. 5. Effects of dexamethasone on proteoglycan core protein mRNA in RMC. RMC were treated with dexamethasone (1 μmol/L) for the indicated times, and levels of (*A*) decorin, (*B*) biglycan, (*C*) versican, and (*D*) perlecan mRNA were assayed by RT-PCR and Northern blot analysis. Upper panels show representative image of Northern blot assay; middle panels are representative images of RT-PCR; lower panels show the results of laser densitometric quantitation of results from the RT-PCR assay. Results shown are the mean ± SEM ($N = 4$ per assay point). $^*P < 0.05$, $^{**}P < 0.01$ vs. control. (*E*) Effects of dexamethasone on decorin and biglycan core protein expression in HMC. HMC were treated with or without dexamethasone (Dex, 1 μmol/L) for 48 hours, and levels of secreted decorin and biglycan were examined by Western blot analysis as described in the **Methods** section.

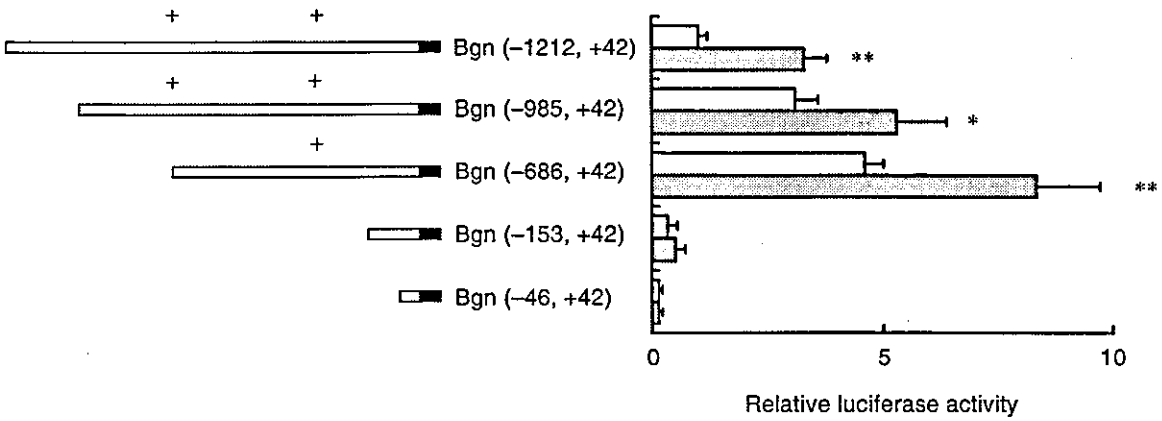

Fig. 6. Effects of dexamethasone on biglycan promoter activity in RMC. RMC were transfected with the indicated biglycan promoter-luciferase constructs, then treated with (▨) or without (□) dexamethasone (1 μmol/L) as described in the **Methods** section. Results shown are the mean ± SEM ($N = 4$ per assay point). $^*P < 0.05$, $^{**}P < 0.01$ vs. Dex (–).

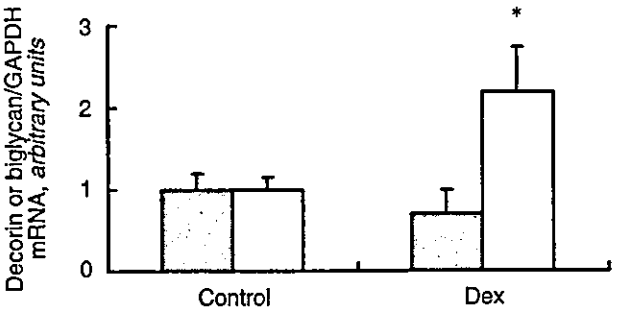

Fig. 7. Effects of in vivo dexamethasone treatment on decorin (▨) and biglycan (□) mRNA levels in isolated glomeruli. Sprague-Dawley rats were treated with daily intraperitoneal injections of dexamethasone (5 mg/kg/day) for 3 days, then glomeruli were isolated, and levels of decorin and biglycan mRNA were assayed by RT-PCR as described in the **Methods** section ($N = 6$). *$P < 0.05$ vs. control.

nephropathy [6, 24]. Of interest, Vleming et al reported that proteoglycan expression in the kidney was a predictor of the severity of renal failure [9]. Taken together, these results suggest that proteoglycans are intimately involved in the pathogenesis of glomerular disease.

Since treatment with steroids plays a central role in the therapy of glomerulonephritis, we examined the regulation of proteoglycans by glucocorticoid hormones. We found that dexamethasone reduced total proteoglycan synthesis in both rat and human cells via a glucocorticoid receptor-mediate pathway. These results were not specific to dexamethasone and were seen also with corticosterone, which is the major natural glucocorticoid in the rat, as well as the therapeutic steroid hormone prednisolone, which is widely used for the treatment of glomerulonephritis.

Biochemical studies using both conventional column chromatography as well as enzyme digestion studies suggested that dexamethasone reduced synthesis of proteoglycans of the CS/DSPG class. Comparison of the molecular size of CS/DSPG produced by mesangial cells with and without dexamethasone treatment did not reveal a major difference in their size or charge, suggesting that the reduced synthesis of GAG was not caused by a reduction in the length of the GAG side-chains, but by a reduction in the number of proteoglycan molecules.

Although the renal glomeruli contain multiple cell types (endothelial cells, mesangial cells, and epithelial cells), it has been shown both biochemically and histologically that the major site of CS/DSPG production is the mesangial cell [25–27]. Thus, the changes brought about by glucocorticoids in the mesangial cell can be assumed to have a major effect on the glomerular extracellular matrix as a whole.

To further investigate the mechanisms of the changes, levels of mRNA for the major proteoglycans expressed by mesangial cells were examined. In the case of the CS/DSPG decorin, a significant decrease in mRNA levels was induced by dexamethasone. In contrast, the levels of biglycan mRNA were unexpectedly increased. To corroborate these findings, Western blot analysis of the biglycan and decorin core proteins was performed, with consistent results. Moreover, in vivo studies confirmed that dexamethasone treatment up-regulates glomerular biglycan gene expression.

What is the significance of the dexamethasone-induced changes in decorin and biglycan expression? Both decorin and biglycan are CS/DSPG, which belong to the family of proteoglycans of relatively small molecular size that contain characteristic leucine-rich repeats and are referred to as SLRPs (small leucine-rich proteoglycans) [28]. These proteoglycans have a variety of important functions, including the control of collagen deposition through interactions with multiple collagens including collagen type I, type V, type VI and type XIV [29–31]. These interactions may be critical in a number of biological processes, such as maintenance and assembly of the collagenous scaffold of the extracellular matrix during growth, development, and wound healing. Indeed, targeted deletion of the decorin and biglycan leads to developmental abnormalities and weakness in tissues containing high amounts of collagen, such as skin and bone [32, 33]. In contrast, over-expression of proteoglycans is found not only in glomerular disease, but also in other disease states such as atherosclerosis [34]. Thus, the glucocorticoid-induced down-regulation of mesangial proteoglycans could serve to attenuate pathological accumulation of extracellular matrix components during glomerular disease.

The increase in biglycan, occurring concomitantly with a decrease in total proteoglycan synthesis, is of particular interest. Although the functions of this proteoglycan have not yet been fully characterized, it is known that biglycan, by virtue of its two negatively-charged GAG side-chains, can bind to, and potentially inactivate transforming growth factor (TGF)-β [35]. TGF-β is a growth promoting cytokine that may play a central role in the pathogenesis of glomerular sclerosis by acting as a common intermediary through which initial injuries of different forms (immunologic, metabolic, and mechanical) can lead to the increased collagen deposition and glomerular sclerosis characteristic of scarred kidneys. Of interest, TGF-β itself causes up-regulation of biglycan, and this is thought to act as an important negative feedback mechanism to inhibit over-action of this growth factor [36]. In view of these reports, the glucocorticoid-induced increase in biglycan expression could be of therapeutic benefit in inhibiting TGF-β and thus attenuating the final common pathway leading to the progression of renal disease.

In this study, we further characterized the molecular mechanisms of the glucocorticoid-induced changes in

biglycan expression. The 5′-flanking regions of the biglycan gene in humans and mice have been characterized and shown to have 90% homology [20]. Both promoter regions are GC rich, contain multiple putative transcription start sites, and multiple regulatory elements. These include activator protein-1 (AP-1) and AP-2 sites, interleukin-6 responsive elements, nuclear factor-κB sites, and E-boxes. Of interest, two sequences characteristic of glucocorticoid responsive elements (GREs) have been located in the 5′-flanking region. Consistent with the sequence data, transfection of the promoter constructs followed by dexamethasone treatment caused an increase in promoter activity that was not seen in the shorter constructs lacking the GREs. These results support the view that glucocorticoids up-regulate biglycan gene expression in mesangial cells by transcriptional control mediated by GREs in the 5′-flanking region of the biglycan gene.

How can we reconcile the data that dexamethasone caused a decrease in the total number of synthesized proteoglycan molecules, with the results suggesting selective up-regulation of biglycan? In the case of decorin, a significant down-regulation of this proteoglycan was seen after dexamethasone treatment, which will have contributed to the net reduction in proteoglycan production. In the case of versican mRNA, a minor decrease was seen that did not attain statistical significance but may have contributed further to the reduction in total proteoglycan synthesis. In support of this view are the data from DEAE-Sephacel chromatography that show a shoulder after peak II (probably corresponding to versican) that appears to be reduced after dexamethasone treatment. A third possibility is that a distinct proteoglycan of the CSPG class but different than versican (one example is the basement membrane CSPG described by Thomas et al [3]) was also markedly down-regulated by dexamethasone treatment.

In contrast to the marked changes in CS/DSPG production, HSPG synthesis was relatively unchanged by glucocorticoid treatment. Proteoglycans of the HSPG class are known to be an integral structural and functional component of the glomerular basement membrane. Concerning the functions of HSPG in the renal glomeruli, multiple laboratory studies have suggested that the strong negative charge carried by the heparan sulfate side chains of the HSPGs in the glomerular basement membrane plays an important role in the formation of the negative charge barrier that maintains glomerular permselectivity and prevents proteinuria [2]. Of interest, glomerular expression of HSPG perlecan has been reported to be decreased in human renal diseases characterized by proteinuria and responsive to steroids, including minimal change disease, membranous glomerulonephritis, and lupus nephritis [2]. Although it is known that HSPGs produced by the mesangial cell may contribute to the composition of the glomerular basement membrane, our results do not support the view that glucocorticoids act therapeutically by causing major changes in the production of HSPG by the mesangial cell.

In summary, the results of this study demonstrate that glucocorticoids can affect glomerular proteoglycan synthesis in vitro and in vivo, and cause distinct changes in proteoglycan core protein expression by mechanisms involving changes in gene transcription. Since proteoglycans play an important role in the progression of renal disease, these results are of interest for understanding some of the mechanisms of actions of glucocorticoids in renal tissues, which would help in designing newer and better strategies for more effective treatment of glomerulonephritis.

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A(-20)C polymorphism of the angiotensinogen gene and progression of IgA nephropathy

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A(-20)C polymorphism of the angiotensinogen gene and progression of IgA nephropathy.

Background. The M235T polymorphism of the angiotensinogen gene (*AGT*) is associated with an increased risk of primary hypertension, which may then lead to progressive renal disease. Recent studies showed that nucleotide substitution in the 5′ upstream core promoter region of *AGT* affects the basal transcription rate of the gene.

Methods. To evaluate the role of *AGT* polymorphisms in the progression of IgA nephropathy (IgAN), we analyzed the association of A(-20)C and M235T polymorphisms with renal prognosis in histologically-proven IgAN patients using the Kaplan-Meier method and Cox proportional hazards regression model.

Results. The incidence of hypertension during the course was associated with T235, but not with C(-20). The renal survival rate for 137 patients with creatinine clearance (C_{Cr}) of 70 mL/min or greater at the time of renal biopsy, and follow-up time of two years or more was significantly lower in the patients with C(-20) ($P = 0.008$). The Cox proportional hazards regression model showed an increased hazard ratio (HR) for urinary protein (more than 2 g/day) of 28.3 (95% CI, 7.3 to 109.8; $P < 0.001$), hypertension at the time of renal biopsy of 4.6 (95% CI, 1.8 to 11.9; $P = 0.002$), and C(-20) of 3.6 (95% CI, 1.5 to 8.7; $P = 0.004$).

Conclusion. This work provides evidence that the C(-20) polymorphism of *AGT*, a subset of T235 alleles, is associated with progression of renal dysfunction in IgAN.

Immunoglobulin A nephropathy (IgAN) is the most common glomerulonephritis among patients undergoing renal biopsy throughout the world. It is characterized by mesangial proliferative glomerulonephritis with predominant IgA deposits. The actuarial renal survival at 10 years is assumed to range between 80% and 85% from apparent onset [1]. Familial clustering of IgAN and inter-individual differences in the clinical course suggests that genetic factors may contribute to the development and progression of this disease.

Previous studies provided definitive evidence that angiotensinogen gene (*AGT*) variants are important in the pathogenesis of cardiovascular diseases such as hypertension. Changes in the 5′ upstream core promoter region of *AGT*, which is essential for the transcription of angiotensinogen mRNA, may cause functional differences that may contribute to pathogenesis [2]. One mutation in particular, an adenine-to-cytosine transition at nucleotide -20 of the 5′ upstream core promoter region [A(-20)C] has been shown to increase the basal promoter activity of *AGT* by increasing the affinity of adenoviral major late transcription factor (MLTF) to this region of the promoter [3].

The existence of an association between *AGT* polymorphisms and the progression of IgA nephropathy is a controversial issue. Pei et al showed that patients with the *AGT* 235MT and TT genotypes have a faster rate of deterioration in creatinine clearance (C_{Cr}) than those with the MM genotypes [4]. However, whether variations in the core promoter region of *AGT* are associated with an actuarial long-term renal prognosis in patients with IgAN is yet to be fully investigated.

METHODS

Patients

Patients were recruited from Niigata University Hospital (Niigata, Japan) as well as other hospitals in the Niigata prefecture. The ethics committee of each institute approved the study. Informed consent was obtained from all participants in the genetic studies.

IgAN was diagnosed by renal biopsy as a mesangial proliferative glomerulonephritis with predominant IgA

Key words: mesangial proliferative glomerulosclerosis, progressive renal disease, glomerulonephritis, hypertension, M235T.

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and C3 depositions in the mesangium. Henoch-Schönlein purpura and secondary IgAN as hepatic glomerulosclerosis were excluded from the analysis.

To analyze the renal survival rate, 137 IgAN patients with a C_{Cr} level of 70 mL/min or greater and a follow-up time of two years or greater were studied. Patients whose C_{Cr} value was less than 70 mL/min at the time of the renal biopsy were excluded because there may have been considerable differences in the onset of IgAN in these patients. Patients whose follow-up time was less than two years also were excluded in order to eliminate the influence of factors other than glomerulonephritis itself. Clinical characteristics including age, sex, duration of observation (in months), body mass index (BMI; kg/m^2), level of urinary protein excretion (g/day), serum creatinine (S_{Cr}; mg/dL), and C_{Cr} (mL/min) were investigated in these patients. Hypertension was defined by the use of one or more antihypertensive medications and/or a blood pressure greater than or equal to 140 mm Hg systolic or 90 mm Hg diastolic blood pressure. The primary end point was defined as the date at which S_{Cr} levels doubled after the time of diagnosis, or when patients underwent their first hemodialysis. For statistical analysis, patients with C(-20) ($N = 55$) were compared with those without C(-20) ($N = 82$) for age, sex, BMI, blood pressure, proteinuria, S_{Cr}, C_{Cr} at the time of renal biopsy, and medical therapy.

A similar analysis was performed in subgroups of the patients with C_{Cr} levels of 70 mL/min or greater who were followed for more than three ($N = 120$) or five years ($N = 92$).

DNA analysis

Genomic DNA from each patient was prepared from peripheral leukocytes in blood samples using an automatic DNA isolation system (NA-100; Kurabo, Osaka, Japan).

To determine the A-C transition at nucleotide -20 of the 5′ upstream region of the core promoter of the *AGT* gene, the following primers were constructed: 5′-primer, 5′-AGAGGTCCCAGCGTGAGTGTC-3′ (nucleotides −166 to −144); 3′-primer, 5′-AGCCCACAGCTCAGT TACATC-3′ (nucleotides 81 to 101) [5]. Polymerase chain reaction (PCR) was performed in a final volume of 50 μL containing 100 ng DNA, 10 pmol of each primer, 250 mmol/L of each of the four dNTPs, 1.5 mmol/L $MgCl_2$, 50 mmol/L KCl, 10 mmol Tris-HCl at pH 8.4, and 2 U of *Taq* polymerase (Takara, Shiga, Japan). The PCR conditions were as follows: 30 cycles of 94°C for 30 seconds, 64°C for one minute, and 72°C for one minute. After PCR, 265-bp products including the 5′ upstream core promoter region were obtained. Then, 8.5 μL of the unpurified product was digested with 2 U of *Eco*O109I (Takara) for at least three hours at 37°C. These samples were separated by 3% agarose gel electrophoresis, and visualized by ethidium bromide staining.

The M235T variant of *AGT* at exon two was determined as described previously [6].

Statistical analysis

Pair-wise linkage disequilibrium (LD) coefficients were estimated by the maximum-likelihood method and the extent of disequilibrium was expressed as $D' = D/D_{max}$ or D/D_{min}, according to Thompson et al [7]. Haplotype frequencies for pairs of alleles were estimated using the Estimating Haplotype-Frequencies software program (ftp://linkage.rockefeller.edu/software/eh).

Statview 5.0J software (SAS Institute, Inc., Cary, NC, USA) was used for statistical analysis. Continuous variables were expressed as mean ± SD or percentage according to clinical features. When the baseline characteristic was continuous (age, disease duration, BMI, urinary protein, S_{Cr}, C_{Cr}), the unpaired t test and Mann-Whitney U test were used. The χ^2 test was used when indicated. The Kaplan-Meier method and the Cox proportional hazards regression model analyzed the time course from renal biopsy to the end point (initiation of dialysis or when the S_{Cr} level doubled after the time of diagnosis). In the Cox regression model, we tested covariates [age, sex, BMI, urinary protein, the category of hypertension, steroid therapy, and administration of angiotensin-converting enzyme inhibitor (ACEI), and the gene polymorphism] by a stepwise backward method and several covariates were selected. The effects of these covariates were expressed by a hazard ratio. A P value less than 0.05 was considered statistically significant.

RESULTS

The genotype distribution in this study was not different from that in Hardy-Weinberg equilibrium. The genotype and allele frequencies of *AGT* M235T and A(-20)C did not differ from previously reported in Japanese studies [5, 8–10]. Haplotype analysis showed a LD between these two alleles (LD coefficient: D′, 1.00). Because the *AGT* variant at -20 was observed only in a subset of the 235T alleles, the following haplotypes were determined: T235 & C(-20); T235 & A(-20); and M235 & A(-20) (Table 1).

Clinical characteristics of the patients investigated are listed in Table 2. A comparison between patients either homozygous or heterozygous for C(-20) and those without C(-20) showed no significant differences in age, sex, BMI, S_{Cr}, C_{Cr}, urinary protein excretion, blood pressure at the time of renal biopsy, or in the percentage of cases treated by ACEI. The percentages of patients administered an antihypertensive agent were not different between the patients with C(-20) and those without C(-20).

Table 1. Genotype, allele, and haplotype frequencies

Genotype distribution	M235T		A(-20)C	
	MM	2	AA	82
	MT	41	AC	49
	TT	94	CC	6
Allele frequency	M235T		A(-20)C	
	M	0.16	A	0.22
	T	0.84	C	0.78
Haplotype frequency				
	T235 & C(-20)		0.22	
	T235 & A(-20)		0.62	
	M235 & A(-20)		0.16	

Glucocorticoids were administered significantly more frequently in patients with C(-20) than those without C(-20).

Because polymorphisms of *AGT* were reported to be associated with essential hypertension in a previous study, the frequencies of genotype M235T were compared between hypertensive and normotensive subjects at the time of renal biopsy and during the observation period (Table 3). The frequency of TT235 was significantly higher in the patients with hypertension during the clinical course and significantly associated with the number of antihypertensive drugs used during the study period. To determine whether C(-20) and the haplotype including C(-20) are associated with hypertension, the frequencies of genotype A(-20)C and the haplotype T235 & C(-20), T235 & A(-20) and M235 & A(-20) were compared between hypertensive and normotensive subjects. C(-20) was not associated with any category of hypertension. The significant increase of M235 & A(-20) in normotensive subjects was observed, which reflected the symmetrical decrease of T235 allele; however, no significant difference in the frequencies of the haplotype T235 & C(-20) between hypertensive and normotensive subjects was observed in both categories.

To examine the effect of A(-20)C polymorphism on disease progression, we compared the survival rate from renal biopsy to the end point in those patients with C_{Cr} level of 70 mL/min or greater at renal biopsy and a follow-up time of two years or more. The renal survival rate in patients with C(-20) was significantly less (χ^2 = 7.0, P = 0.008) than in patients without C(-20) (Fig. 1). Moreover, in patients with a C_{Cr} level of 70 mL/min or greater at renal biopsy and follow-up time of more than three years (N = 120; mean observed periods, 116.4 months) or five years (N = 92; mean observed periods, 137.5 months), the renal survival rate was significantly lower in patients with C(-20) (P = 0.02 at 3 years and P = 0.04 at 5 years). The renal survival rate in TT235 patients (N = 43) also was significantly lower than in MM/MT235 patients (N = 94; P = 0.02; Fig. 2).

The Cox proportional hazards regression model showed an increased hazard ratio (HR) for C(-20), 3.6 (95% CI, 1.5 to 8.7; P = 0.004) from multivariate analysis, including several covariates selected by stepwise backward analysis (hypertension at the time of renal biopsy, proteinuria more than one or two grams per day; Table 4). The HR for urinary protein more than two grams per day (vs. <1 g/day) was extensively increased, which was 28.3 (95% CI, 7.3 to 109.8; $P < 0.001$) from multivariate analysis. Hypertension at the time of renal biopsy was demonstrated to be a statistically significant risk factor in multivariate analysis including urinary protein and C(-20) (HR 4.6; 95% CI, 1.8 to 11.9; P = 0.002).

DISCUSSION

This study demonstrated that the renal survival rate was significantly lower in patients with C(-20) in Japanese patients with IgAN. The Cox proportional hazards regression model showed an increased hazard ratio, 3.6 in multivariate analysis, indicating that this polymorphism is an independent risk factor for progression to end-stage renal failure.

Previously, association studies of *AGT* A(-20)C and essential hypertension in the Japanese population were reported [5, 8–10]. However, to our knowledge an association of the *AGT* A(-20)C polymorphism with the progression of IgAN was not investigated. Our study clearly demonstrates that C(-20) is an independent risk factor for the progression of IgAN in patients whose renal function was preserved at the time of renal biopsy. Many confounding factors are known to affect the progression of IgAN, including immune-mediated events [11–13], hemodynamic factors [14, 15], cell proliferation and an increase in extracellular matrix [12, 16, 17]. In this study, systemic hypertension may play a role in the decline of renal function in patients with *AGT* TT235, because the TT235 was significantly associated not only with hypertension during the clinical course, but also with the renal prognosis. These observations were assumed to reflect the influence of A(-6), which has been known to increase the level of transcription of angiotensinogen, because A(-6)G and M235T polymorphism of *AGT* are in complete LD [18, 19]. In contrast and unexpectedly, C(-20) was significantly associated with renal prognosis independently of hypertension. Although the exact mechanism that explains these dissociated results on A(-20)C and M235T polymorphisms remained unclear, these results suggest that the transcriptional regulation of *AGT* in the renal tissue is distinct from the systemic circulation. It has been reported that angiotensin II in renal interstitial fluids are much higher than plasma levels, suggesting the compartmentalization and independent regulation of renal angiotensin II [20]. Furthermore, recent haplotype studies demonstrated that LD between M235T and A(-20)C was not more complete than M235T and A(-6)G [18, 21],

Table 2. Clinical characteristics at the time of renal biopsy

	All patients (N = 137)	*AGT* AA(-20) (N = 82)	*AGT* AC/CC(-20) (N = 55)
Observed periods *months*	105.7 ± 68.3	104.6 ± 69.4	107.2 ± 67.1
Background 1st renal biopsy			
Age *years*	34.9 ± 12.4	35.8 ± 12.8	33.5 ± 11.7
Male %	45.3	43.9	47.3
BMI kg/m^2	22.5 ± 2.8	22.9 ± 3.0	22.0 ± 2.4
U_{prot} *g/day*	1.2 ± 1.1	1.1 ± 0.7	1.4 ± 1.4
S_{Cr} *mg/dL*	0.8 ± 0.2	0.8 ± 0.2	0.8 ± 0.2
C_{Cr} *mL/min*	104.6 ± 23.5	106.6 ± 24.8	101.6 ± 21.2
Blood pressure *mm Hg*			
Systolic	125.8 ± 16.9	126.3 ± 18.0	125.1 ± 15.1
Diastolic	75.6 ± 12.6	76.4 ± 13.0	74.4 ± 12.0
Treatment during the course			
Glucocorticoid %	24.3	16.0	36.4*
Antihypertensive drugs %	53.3	47.6	61.8
ACEI %	40.1	39.0	41.8

Abbreviations are: BMI, body mass index; U_{prot}, urinary protein; S_{Cr}, serum creatinine; C_{Cr}, creatinine clearance; ACEI, angiotensin-converting enzyme inhibitor.
*$P < 0.05$ vs. *AGT* AA(-20) patients

Table 3. Genotype and haplotype frequencies in hypertensive and normotensive subjects

	At the renal biopsy			During the course			Number of anti-HT drugs	
Genotype	HT %	NT %	P	HT %	NT %	P	mean ± SD	P
MM/MT235	6.6	24.8		12.4	19.0		0.7 ± 1.1	
TT235	25.5	43.1	0.08	47.4	21.2	0.001	1.2 ± 1.3	0.003
AA(-20)	19.7	40.1		32.8	27.0		1.0 ± 1.3	
AC/CC(-20)	12.4	27.7	0.85	27.0	13.1	0.16	1.1 ± 1.2	0.36

	At the renal biopsy			During the course		
Haplotype	HT %	NT %	P	HT %	NT %	P
T235 & C(-20)	21.6	22.6	0.49	25.0	18.0	0.12
T235 & A(-20)	69.3	58.1	0.048	64.6	57.3	0.14
M235 & A(-20)	9.1	19.5	0.02	10.4	24.5	0.002

Abbreviations are: HT, hypertensives; NT, normotensives; P, P value.

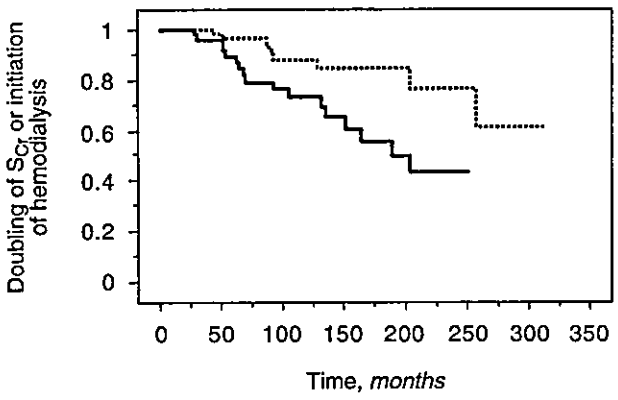

Fig. 1. *AGT* A(-20)C and the renal survival rate in patients with IgAN. The renal survival rate in patients with C(-20) (N = 55) was less than that in patients without C(-20) (N = 82). Symbols are: (dotted line) AA(-20); (solid line) AC/CC(-20). Log-rank test, P = 0.008.

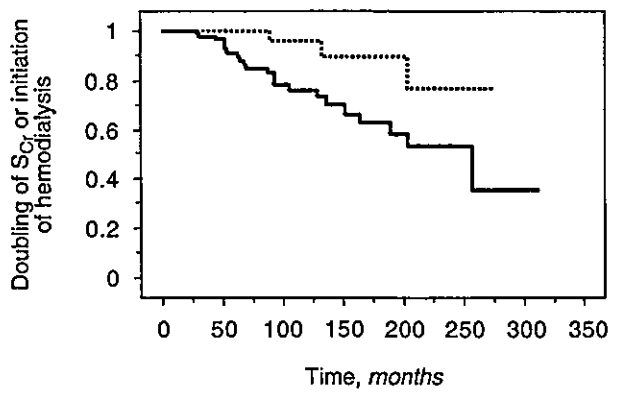

Fig. 2. *AGT* M235T and the renal survival rate in patients with IgAN. The renal survival rate in patients with MM/MT235 (dotted line; N = 94) was less than that in patients with TT235 (solid line; N = 43). Log-rank test P = 0.02.

Table 4. Cox proportional hazards regression model

Variable	*P* value	HR	95% CI
U_{prot}			
>1 g/day, <2g/day	0.01	5.4	1.5 to 20.4
>2 g/day	<0.001	28.3	7.3 to 109.8
HT at renal biopsy	0.002	4.6	1.8 to 11.9
AGT C(-20)	0.004	3.6	1.5 to 8.7

Abbreviations are: U_{prot}, urinary protein; HT, hypertension; HR, hazard ratio; CI, confidence interval.

although C(-20) is a subset of T235. There is a possibility that the A(-20)C polymorphism may be chiefly related to the local activation of renin-angiotensin system through a different transcriptional regulation, leading to renal dysfunction, whereas the M235T polymorphism may be implicated with renal dysfunction through systemic hypertension. Further study is necessary to explore the molecular mechanism of transcriptional regulation of *AGT* in the kidney under both physiological and pathological conditions.

Several earlier studies have analyzed an association of T235 variants of *AGT* with the progression of IgAN. Pei et al showed that patients with MT and TT genotypes had a faster rate of deterioration of renal function than those with MM genotypes [4]. Because a large proportion of patients in their study were treated with antihypertensive drugs (56 to 82%) and renal function in patients with MT or TT was moderately impaired, the effects of this polymorphism might be directly on blood pressure rather than having an independent effect on deterioration of renal function. In contrast, Hunley et al failed to find an association between the *AGT* T235 variant and any clinical categories of deterioration in IgAN [22]. However, in their study the length of clinical observation (6 to 7 years) appeared to be short for classifying the patients into categories. "Observation bias" would tend to misclassify the patients who may be destined to be in the disease progression group, to the stable renal function group [23]. Recently, a large and well-designed study (IGARAS) investigated the role of renin-angiotensin system gene polymorphisms in the progression of IgAN [24]. In their study, the distribution of *AGT* M235T genotypes was not different among the patients grouped by S_{Cr} and proteinuria at the time of renal biopsy. The Cox proportional hazards regression model did not find predictive values of *AGT* polymorphisms for renal survival; however, information about the long-term effect of each polymorphism on the progression of IgAN with preserved renal function at the time of renal biopsy was not available.

The limitation of this study may be that the patients who had different degrees of renal injury and had different rates of progression of renal dysfunction were recruited, although we selected the patients whose C_{Cr} at the time of renal biopsy was more than 70 mL/min. Histopathological analysis of the patients who reached end points within several years revealed severe expansion of mesangial matrix, tuft adhesion, and crescent formation. However, the mean time to the end points in our study was nearly 10 years, and a substantial proportion of the patients had stable or slowly declining renal function during the follow-up period. D'Amico et al's study found that the speed of progression of end-stage renal failure in IgAN patients was quite variable [25]. In fact, there was a large inter-individual difference in the time course to reach the end point, which was the very point we investigated here as the genetic background. We employed a time-to-event analysis because it is suggested that, in a study including patients with stable renal function, an analysis of the time-to-event approach is favored over an analysis of mean slope of renal function [26].

Our study could not provide evidence that the *AGT* C(-20) influenced the therapeutic effect of angiotensin II blockade by ACEI. In this respect, further investigation with a long-term prospective observation of a large number of cases is necessary. However, our study suggests that genotyping of *AGT* at -20 and precise clinicopathological assessments can lead to more accurate estimations of the prognosis, and to more proper and active usage of ACEI or ARB in patients with IgAN.

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Smad7 mediates transforming growth factor-β–induced apoptosis in mesangial cells

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Smad7 mediates transforming growth factor-β–induced apoptosis in mesangial cells.

Background. In addition to inhibiting cell growth, transforming growth factor-β (TGF-β) has recently been reported to induce apoptosis in various cell lines. Smad proteins are the downstream effectors of TGF-β signaling. Among them, Smad7 exerts negative feedback control over the action of TGF-β. However, we do not know how the Smad proteins contribute to TGF-β–induced apoptosis in mesangial cells. To investigate the function of Smad proteins, we examined the effect of Smad overexpression using adenoviral vector in mesangial cells.

Methods. Primary cultured rat mesangial cells were transfected with Smad7-promoter-luciferase-plasmid by electroporation. Smad7 promoter activity was investigated by luciferase assay. The apoptotic phenomena elicited by TGF-β and Smad7 overexpression were investigated using adenoviral vector (AdCMV-Smad7). Apoptosis was detected by the cell death detection ELISA assay, CPP32/caspase-3 assay, and nucleosomal DNA laddering.

Results. TGF-β significantly increased the protein expression and the promoter activity of Smad7 in rat mesangial cells. Overexpression of Smad7 induced DNA fragmentation and significant increases in cell death ELISA and CPP32/caspase-3 assay. On the other hand, overexpression of Smad2 and Smad3 did not elicit any significant increases in CPP32/caspase-3 activity. Furthermore, the antisense oligonucleotide to Smad7 prevented the TGF-β–induced apoptosis. Overexpression of Smad7 did not affect nuclear factor-κB activity in mesangial cells.

Conclusions. These data indicate that TGF-β–induced apoptosis in mesangial cells is mediated through the activation of caspase-3 by Smad7, but not by Smad2 or Smad3. Our results provide new clarification on the function of Smad7 in TGF-β signaling in mesangial cells.

Key words: cell death, TGF-β, signal transduction, caspase, nuclear factor-kappa B, adenoviral vector, sclerosis, glomerulus.

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In glomerular disease, mesangial cell proliferation is an important pathology that can potentially progress to sclerosis and end-stage renal disease [1, 2]. Glomerular mesangial cells are thought to play a key role in promoting glomerular scarring.

Apoptosis is very important for homeostasis in the formation of various diseases. Mesangial cells have also been observed to undergo apoptosis in various glomerular diseases [3], and recent experimental evidence suggests that this apoptosis of mesangial cells mediates the resolution of glomerular hypercellular injury and plays a critical role in the regulation of cellularity following renal injury [4, 5]. Apoptotic phenomena were observed not only in experimental glomerulonephritis, but also in human glomerular diseases such as IgA nephropathy and lupus nephritis [6]. The authors of these studies suggested that apoptosis was at least partially involved in the cell deletion of various progressive glomerular diseases.

Transforming growth factor-β (TGF-β) affects many functions in many different types of cells [7, 8]. TGF-β exerts manifold effects on mesangial proliferation, promoting it at lower concentrations and suppressing it at higher concentrations [9]. In a model of antithymocyte serum-induced acute mesangial proliferative glomerulonephritis, TGF-β production promoted the accumulation of glomerular extracellular matrix [10]. Likewise, in a model of anti-glomerular basement membrane antigen-induced crescentic glomerulonephritis, antiserum against TGF-β suppressed experimental glomerulonephritis [11]. Thus, TGF-β is known to be involved in the mesangial proliferation in various types of nephritis. Separate experiments also have demonstrated that TGF-β induces apoptosis in mesangial cells [12], although there has been little clarification of the pathway of this TGF-β–induced apoptosis.

Smads are the signal transducers for the members of the TGF-β superfamily [13]. TGF-β stimulates Smad7, and the stimulated proteins take part in a cycle of negative feedback control over the action of TGF-β [7]. An

analysis of Smad protein expression is expected to provide key information on the pathway of TGF-β–induced apoptosis in mesangial cells. While several recent articles have reported that Smad7 regulates apoptosis, the overall effect of this protein on apoptosis remains controversial. Smad7 inhibited TGF-β–induced apoptosis in B cells [14], M1 cells, and Hep3B cells [15], whereas it was found to mediate TGF-β–induced apoptosis in prostatic carcinoma cells [16], Mv1Lu cells, Madin-Darby canine kidney (MDCK) cells [17], and podocytes [18]. Moreover, a recent report showed that TGF-β and Smad7 induced apoptosis in podocytes through different downstream pathways, providing a novel molecular mechanism for podocyte depletion in progressive glomerulosclerosis [18]. While the apoptosis in endocapillary/mesangial cells was shown to be significantly increased at the advanced stage of glomerulosclerosis in TGF-β transgenic mice, the report did not go into detail on how Smad7 and TGF-β contribute to apoptosis in mesangial cells.

Studies on various cell-lines have confirmed that TGF-β and receptor-activated Smads (R-Smads) such as Smad3 stimulate inhibitory Smads (I-Smads), and also that the Smad signaling system exerts an auto-regulatory action via a negative feedback loop [19–21]. However, there has been no clear data on primary mesangial cells.

By examining the effect of Smad7 overexpression using adenovial vector in cultured primary rat mesangial cells, we determined that Smad7 overexpression induced apoptosis, and that the TGF-β–induced apoptosis was mediated by Smad7 rather than Smad2 or Smad3.

METHODS

Recombinant adenoviruses

The recombinant adenovirus expressing the *LacZ* gene (AdCMV-LacZ) and the replication-defective, recombinant adenoviruses expressing the *Smad7* gene (AdCMV-Smad7) were prepared as described previously [22–24]. Recombinant adenoviruses expressing the *Smad2* gene (AdCMV-Smad2) and *Smad3* gene (AdCMV-Smad3) were kindly provided by Dr. K. Miyazono. Each adenovirus preparation was titrated by plaque-assay on 293 cells. Viral stocks [5×10^8 plaque-forming units (pfu)/mL] were stored at −80°C and thawed on ice just before use. The efficiency of adenovirus-mediated gene transfer is over 90% in mesangial cells [23].

Cell culture and preparation of extracts

Mesangial cell strains from male Sprague-Dawley (SD) rats were isolated and characterized as previously reported [25]. Cells were maintained in RPMI 1640 medium supplemented with 20% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 5 μg/mL of insulin, 5 μg/mL of transferrin, and 5 ng/mL selenite at 37°C in a 5% CO_2 incubator. The cells were used in the experiments after 5 to 10 passages. The cells were incubated in medium plus 20% fetal calf serum (FCS) until reaching approximately 80% confluence, then either transfected with adenovirus for 48 hours, or exposed to TGF-β (1 ng/mL) or tumor necrosis factor-α (TNF-α; 10 ng/mL) for 24 hours in medium containing 0.5% FCS.

Caspase-3 assays

A Caspase-3 Fluorometric Protease Assay Kit (MBL, Tokyo, Japan) was used for measurement of caspase-3 activities as previously described [24]. In brief, the cells were plated in six-well dishes, cultured in medium, collected, and lysed in lysis buffer at indicated times. After the protein concentration was normalized by the Bradford assay, the lysates were incubated with the same amounts of reaction buffer and 50 mmol/L DEVD-AFC substrate for two hours at 37°C. Fluorescence was monitored with an excitation wavelength of 400 nm and an emission wavelength of 505 nm.

Cell death ELISA

Histone-associated DNA fragments were quantitated by enzyme-linked immunosorbent assay (ELISA; Boerhinger, Mannheim, Germany). All cells from each well were collected by trypsinization and pipetting, pelleted (800 rpm, 5 min), lysed, and subjected to the capture ELISA according to the manufacturer's protocol. Cytosolic proteins were collected using cell lysis buffer according to the manufacturer's protocol. After a 30-minute incubation of the cells with cell lysis buffer, the samples were centrifuged for 10 minutes (15,000 rpm), the nuclei were formed into pellets, the cytoplasmic fraction became supernatant, and the supernatants were collected for the ELISA assay. Each experiment was carried out in triplicate and repeated independently at least five times.

Ladder assays

Adherent and floating cells were collected and lysed in a medium containing 10 mmol/L Tris (pH 8.0), 100 mmol/L NaCl, and 25 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.5% sodium dodecyl sulfate (SDS), and 1.0 mg/mL proteinase K at 37°C for four hours. DNA was extracted from the digested cells as previously described [26], divided into 30 μg portions, and subjected to electrophoresis on 1.5% agarose gels.

Antisense oligonucleotide

Antisense and scrambled random sequence oligonucleotides (oligonucleotide phosphorothioates) against Smad7 were synthesized by a DNA synthesizer (Model 8909; Perseptive Biosystems, Cambridge, MA, USA) as previously described [16, 27]. The Smad7 antisense oligonucleotide was designed around the region 107-127 (5′-GTCGCCCCTTCTCCCCGCAGG-3′) of the rat Smad7 complementary DNA sequence (GenBank acces-

sion number AF159626). The Smad7 sense oligonucleotide was 5′-CCTGCGGGGAGAAGGGGCGAC-3′. Rat mesangial cells were exposed to TGF-β treatment (1 ng/mL) or TGF-β treatment with antisense oligonucleotide to Smad7 (AS) or TGF-β treatment with sense oligonucleotide to Smad7 (sense) for 24 hours.

Transient transfection and luciferase assay

Rat mesangial cells were transfected by the electroporation method. The Smad7-promoter luciferase construct was kindly provided by Dr. Y. Chen [21]. Data are representative of at least four independent experiments performed in duplicate, and expressed as the *N*-fold increase in luciferase activity calculated relative to the indicated level of Smad7 promoter activity. Plasmid DNAs (10 μg) were transfected by the electroporation method. For experiments performed in exponentially growing cells, the Smad7-promoter luciferase activity was measured 48 hours after transfection.

The plasmids were nuclear factor-kappa B (NF-κB)-luciferase-plasmids (NF-κB elements × 5 and luciferase fusion plasmid) as the NF-κB–sensitive promoter from Stratagene (La Jolla, CA, USA). Data are representative of at least four independent experiments performed in duplicate, and expressed as the *N*-fold increase in luciferase activity calculated relative to the indicated level of NF-κB–sensitive promoter activity. Plasmid DNAs (10 μg) were transfected by the electroporation method. In the experiments performed with exponentially growing cells, the cells after the NF-κB plasmids were transfected, incubated in 20% FCS-containing medium for 24 hours, and then either infected with adenovirus for 48 hours in medium containing 0.5% FCS, or incubated for 24 hours and exposed to TNF-α treatment (10 ng/mL) for 24 hours in medium containing 0.5% FCS. The NF-κB–sensitive promoter luciferase activity was measured 48 hours after infection. Normalization was achieved by cotransfecting 3.0 μg of pCH110, a β-galactosidase reporter construct, as an internal control for the transfection efficiency. Luciferase and β-galactosidase activities were measured according to the manufacturer's instructions (Promega, Madison, WI, USA) protocol. Transfection efficiency of electroporation is around 20% in mesangial cells [28].

Western blot analysis

The proteins were extracted from mesangial cells using an extraction buffer containing 20 mmol/L HEPES, pH 7.5, 250 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L egtazic acid (EGTA), 10% glycerol, 0.1% (vol/vol) Nonidet P-40, 0.5 mmol/L dithiothreitol (DTT), 0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 μg/mL aprotinine, and 1 μg/mL pepstatin A, as described previously [28–30]. Lysates were allowed to remain on ice for 30 minutes, and then centrifuged at ×15,000 rpm for 30 minutes at 4°C. The soluble lysates were mixed at a 1:4 ratio with ×5 Laemmli buffer and heated for four minutes at 94°C. Twenty micrograms of protein was resolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to an Immobilon P membrane (Daiichikagaku, Tokyo, Japan), and then probed with the following antibodies: goat polyclonal antibodies against Smad7, Smad2, and Smad3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or a rabbit polyclonal antibody against actin (Santa Cruz Biotechnology) or with rabbit polyclonal antibodies against Ser473-phospho-specific Akt, total-Akt, Ser32-phospho-specific IκB-α, and total IκB-α (Cell Signaling Technology, Inc., Beverly, MA, USA) . The experiments for the detection of Smad7, Smad2, and Smad3 used the primary antibodies (diluted 1/1000) and horseradish peroxidase (HRP)-conjugated anti-goat IgG (diluted 1/2500) as a secondary antibody. The blots for the detection of Ser473-phospho-specific Akt, total-Akt, Ser32-phospho-specific IκB-α, total IκB-α, and actin used the primary antibodies (diluted 1/1000) and HRP-conjugated anti-rabbit IgG (diluted 1/2500) as a secondary antibody. The bands were visualized using the enhanced chemiluminescence (ECL) system (Amersham, Little Chalfort, UK) after extensive washing of the membranes. ECL films were scanned using an Epson scanner (Nagano, Japan).

Controls for protein loading were identified by actin as the internal standard [31, 32].

Electrophoretic mobility shift assay (EMSA)

Nuclear extract from mesangial cells was prepared as described previously, with minor modifications [27]. Firstly, mesangial cells were collected using trypsin and centrifuged. After resuspending the cell pellets in tubes containing 400 μL of ice-cold buffer A (10 mmol/L HEPES, pH 7.9, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L DTT, 0.5 mmol/L PMSF), 25 μL of a 10% Nonidet P-40 solution was added, and the tubes were vigorously shaken for 30 seconds on an orbital shaker and centrifuged for 30 seconds in a microcentrifuge. Next, the nuclear pellets were resuspended in tubes containing 100 μL of ice-cold buffer C (20 mmol/L HEPES, pH 7.9, 400 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 1 mmol/L PMSF, 1 μg/mL aprotinine, and 1 μg/mL pepstatin A), vigorously rocked at 4°C for 15 minutes on a shaking platform, and centrifuged for 10 minutes at 4°C. The collected supernatants were tested by a gel shift assay system according to the manufacturer's protocol (Promega). Briefly, nuclear extracts (10 μg of each) underwent a reaction in a premixed incubation buffer (included in the kit) with γ-^{32}P-end labeled nuclear factor-κB (NF-κB) consensus oligonucleotides (5′-AGTTGAGGGGACTTTCCCAGGC-3′) for 30 minutes at 25°C. To establish the specificity of the reaction, competition assays were performed with 100-fold excess of unlabeled NF-κB consensus, or unrelated

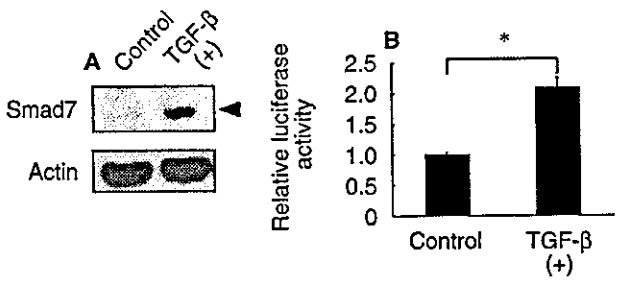

Fig. 1. Effect of transforming growth factor-β (TGF-β) on Smad7 in rat mesangial cells. (*A*) Western blot analysis of Smad7 on cell lysates from rat mesangial cells stimulated with TGF-β. Rat mesangial cells were treated with or without TGF-β (1 ng/mL) for 24 hours. The protein expression of Smad7 was determined by Western blot analysis using 20 μg of cell lysate protein and a specific antibody. (*B*) Smad7-promoter-luciferase activity. Rat mesangial cells were transiently co-transfected with Smad7-promoter-luciferase plasmid and β-galactosidase plasmid, and then treated with or without TGF-β (1 ng/mL) for 24 hours. Luciferase specific activity was determined by luciferase assay. The luciferase activity was normalized by β-galactosidase activity ($N = 6$; data are mean ± SEM; $^*P < 0.001$).

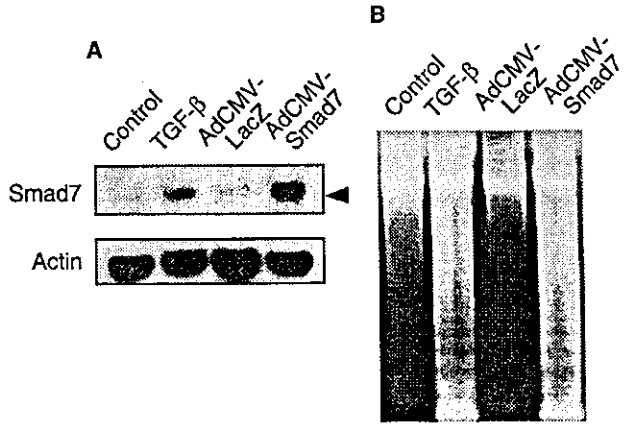

Fig. 2. Adenovirus-mediated Smad7 gene transfer (AdCMV-Smad7) induced nucleosomal laddering of DNA in rat mesangial cells. (*A*) Western blot analysis of Smad7 on cell lysates from mesangial cells. Rat mesangial cells were either transfected with AdCMV-Smad7 or AdCMV-LacZ for 48 hours, or exposed to TGF-β treatment (1 ng/mL) for 24 hours. Twenty micrograms of cell lysate protein were used for Western blot analysis. The protein production of Smad7 was determined by Western blot analysis using a specific antibody. (*B*) DNA ladder assays. Rat mesangial cells were either transfected with AdCMV-Smad7 or AdCMV-LacZ for 48 hours, or exposed to TGF-β treatment (1 ng/mL) for 24 hours. Extracted DNA from adherent and floating cells was subjected to electrophoresis on 1.5% agarose gels.

oligonucleotides (activated protein-1, AP-1; Promega). After the reaction, the samples were analyzed on a 6% nondenaturing polyacrylamide gel. The gel was dried and exposed to x-ray film.

Statistics

The results were given as means ± SEM. The differences were tested using two way-analysis of variance followed by the Scheffe's test for multiple comparisons. Two groups were compared by the unpaired t test. $P < 0.05$ was considered significant.

RESULTS

TGF-β induced Smad7 in mesangial cells

Firstly, to determine the mechanism of Smad7 in primary cultured rat mesangial cells, the effect of TGF-β on the protein expression of Smad7 was examined. Rat mesangial cells were treated with or without TGF-β (1 ng/mL) for 24 hours. The protein expression of Smad7 was determined by Western blot analysis using 20 μg of cell lysate protein and a specific antibody. TGF-β significantly increased the Smad7 expression, as shown in Figure 1A.

In addition, the promoter activity of Smad7 was measured to examine the regulation of Smad7 expression by TGF-β in rat mesangial cells. The mesangial cells were transiently transfected with Smad7-promoter-luciferase-plasmid by the electroporation method.

Compared with the Smad7 promoter-luciferase activity in the controls, the promoter activity increased about 2.1-fold under TGF-β treatment (Fig. 1B).

These results confirmed that TGF-β induced Smad7 in primary mesangial cells.

Smad7 overexpression stimulated apoptosis in mesangial cells

Earlier studies have shown variability in the Smad7 regulation of apoptosis in different cell lines [14–18]. To explore whether Smad7 can induce the apoptotic phenomena in mesangial cells, Smad7 was overexpressed using the adenoviral vector. Primary cultured rat mesangial cells were transfected with adenovirus (AdCMV-Smad7 or AdCMV-LacZ as a control) for 48 hours or exposed to TGF-β treatment (1 ng/mL) for 24 hours.

First, to verify whether an adenoviral system for Smad7 could achieve efficient expression of the Smad7 protein, Smad7 protein expression was determined by Western blot analysis using a specific antibody. As shown in Figure 2A, AdCMV-Smad7 increased the Smad7 expression to a significantly greater extent than AdCMV-LacZ at 48 hours post-transfection.

In the next experiment, using the agarose gel electrophoresis of fragmented DNA, nucleosomal DNA laddering was found to be induced in the cells treated by TGF-β treatment and adenovirus-mediated Smad7 gene transfer, but not in the control cells without adenoviral treatment and the cells exposed to AdCMV-LacZ (Fig. 2B). This meant that the apoptosis in rat mesangial cells was induced not by viral toxicity, but by the overexpression of Smad7.

Next the apoptotic stimulation was quantified using the cell death detection ELISA assay and CPP32/caspase-3 fluorometric protease assay. In the cell death de-

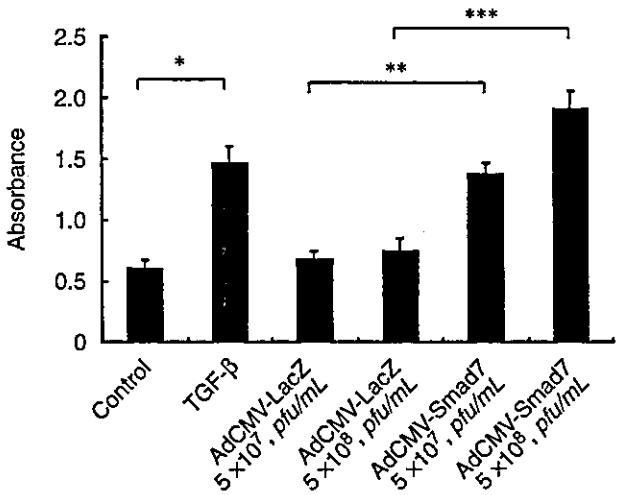

Fig. 3. Smad7-overexpression-stimulated apoptosis, assessed by cell death detection ELISA assays. Rat mesangial cells were transfected with adenovirus (AdCMV-Smad7 or AdCMV-LacZ) for 48 hours, or exposed to TGF-β treatment (1 ng/mL) for 24 hours. Cell lysate from adherent and floating cells was subjected to cell death ELISA assay ($N = 6$; data are mean ± SEM; $^*P = 0.002$, $^{**}P = 0.013$, $^{***}P < 0.001$).

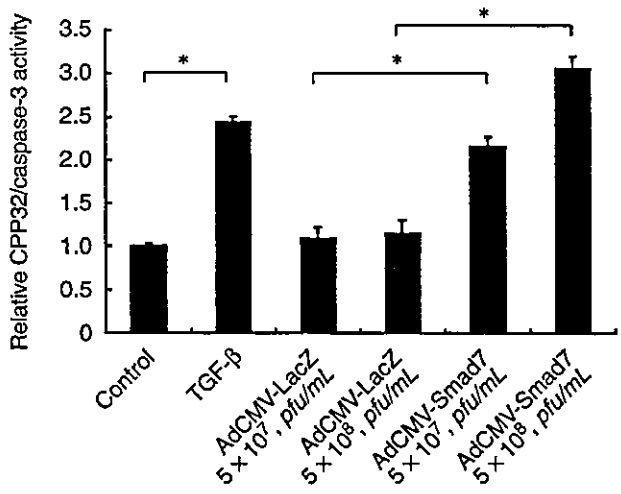

Fig. 4. Smad7-overexpression-stimulated apoptosis, assessed by CPP32/caspase-3 fluorometric protease assays. Rat mesangial cells were transfected with adenovirus (AdCMV-Smad7 or AdCMV-LacZ) for 48 hours, or exposed to TGF-β treatment (1 ng/mL) for 24 hours. Cell lysate from adherent and floating cells was used for CPP32/caspase-3 assay ($N = 6$; data are mean ± SEM; $^*P < 0.001$).

tection ELISA assay, AdCMV-Smad7 induced a significant 2.6 ± 0.2-fold increase in activation compared to AdCMV-LacZ, and the level of apoptosis induced by Smad7 overexpression tended to increase with the dose of adenovirus (Fig. 3). The cell death ELISA examination of mesangial cells exposed to TGF-β showed that the apoptotic signals were significantly increased by 2.4 ± 0.1-fold over the control level.

Similarly, overexpression of Smad7 by adenovirus induced a significant 2.8 ± 0.1-fold increase over the level with AdCMV-LacZ, and TGF-β induced a 2.5 ± 0.1-fold increase in CPP32/caspase-3 activity over the level in controls (Fig. 4). The CPP32/caspase-3 activity induced by Smad7 overexpression also tended to increase with higher doses of adenovirus. On the other hand, overexpression of LacZ did not show an apoptotic increase in CPP32/caspase-3 activity (Fig. 4). These results demonstrated that Smad7 overexpression induced apoptosis in mesangial cells, and that the apoptotic pathways initiated by Smad7 and TGF-β in mesangial cells were related to the caspase cascades and activation of caspase-3.

Smad2 and Smad3 overexpression did not induce increased caspase-3 activity in mesangial cells

After confirming that Smad7 and TGF-β induced apoptosis through the activation of caspase-3 in mesangial cells, we examined whether R-Smads such as Smad2 and Smad3 also were able to stimulate apoptosis in mesangial cells. Primary cultured rat mesangial cells were either transfected with adenovirus (AdCMV-Smad2, AdCMV-Smad3, AdCMV-Smad7, or AdCMV-LacZ) for 48 hours or exposed to TGF-β treatment (1 ng/mL) for 24 hours.

First, to verify whether an adenoviral system for Smad2 and Smad3 could elicit efficient expressions of the Smad proteins, the protein expressions were determined by Western blot analysis using specific antibodies. As shown in Figure 5 A and B, AdCMV-Smad2 and AdCMV-Smad3 increased the expressions of Smad2 and Smad3 respectively, to a significantly greater extent than AdCMV-LacZ at 48 hours post-transfection.

Next, the apoptotic phenomenon was quantified using CPP32/caspase-3 fluorometric protease assay. Compared with the mesangial cells exposed to AdCMV-LacZ infection, the cells exposed to AdCMV-Smad2 and AdCMV-Smad3 did not show a significant increase in caspase-3 activity (Fig. 5C). In contrast, overexpression of Smad7 stimulated a 3.4 ± 0.1-fold increase over the level in AdCMV-LacZ–exposed cells, and TGF-β induced a 2.6 ± 0.1-fold increase in CPP32/caspase-3 activity compared with the control level. These results demonstrated that it was not the R-Smads such as Smad2 and Smad3, but Smad7 itself that increased apoptosis through the caspase cascade along with TGF-β.

The antisense oligonucleotide to Smad7 forestalled TGF-β–induced apoptosis in mesangial cells

The activation of caspases is involved in the apoptotic regulation by both TGF-β and Smad7 overexpression. To investigate the relationship between Smad7 and TGF-β, we tried to block Smad7 expression using the antisense method, and then confirmed whether Smad7 expression

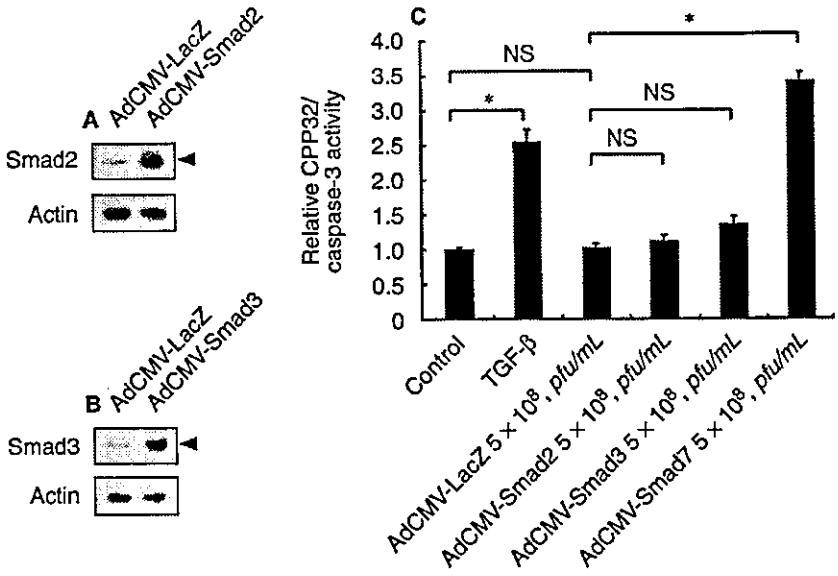

Fig. 5. Smad2 and Smad3 overexpression did not induce significant changes in CPP32/caspase-3 activity. (*A*) Western blot analysis of Smad2 on cell lysates from mesangial cells. Rat mesangial cells were transfected with either AdCMV-Smad2 or AdCMV-LacZ for 48 hours. Twenty micrograms of cell lysate protein were used for Western blot analysis. The protein production of Smad2 was determined by Western blot analysis using a specific antibody. (*B*) Western blot analysis of Smad3 on cell lysates from mesangial cells. Rat mesangial cells was transfected with either AdCMV-Smad3 or AdCMV-LacZ for 48 hours. Twenty micrograms of cell lysate protein were used for Western blot analysis. The protein production of Smad3 was determined by Western blot analysis using a specific antibody. (*C*) Rat mesangial cells were transfected with adenovirus (AdCMV-Smad2 or AdCMV-Smad3 or AdCMV-Smad7 or AdCMV-LacZ) for 48 hours, or exposed to TGF-β treatment (1 ng/mL) for 24 hours. Cell lysate from adherent and floating cells was used for CPP32/caspase-3 assay (N = 6; data are mean ± SEM; $^*P < 0.001$).

was essential for TGF-β–induced apoptosis. Rat mesangial cells were exposed to TGF-β treatment alone (1 ng/mL), to TGF-β treatment with the synthetic antisense oligonucleotide to Smad7, or to TGF-β treatment with the sense oligonucleotide to Smad7 for 24 hours.

According to our Western blot analysis, the Smad7 antisense oligonucleotides reduced the TGF-β–induced increase in Smad7 protein to the control level, whereas the sense oligonucleotide to Smad7 did not (Fig. 6A).

Next, the apoptotic phenomenon was quantified using CPP32/caspase-3 fluorometric protease assay. TGF-β treatment alone and TGF-β treatment with sense to Smad7 induced 2.5 ± 0.1- and 2.0 ± 0.1-fold increases in caspase-3 activity, respectively, whereas antisense Smad7 oligonucleotides did not elicit significant increases compared with controls (Fig. 6B). The effect of the Smad7 antisense oligonucleotides inhibited TGF-β–induced apoptosis in caspase-3 activity to the control level. These results demonstrated that Smad7 mediated the TGF-β–induced apoptosis in mesangial cells.

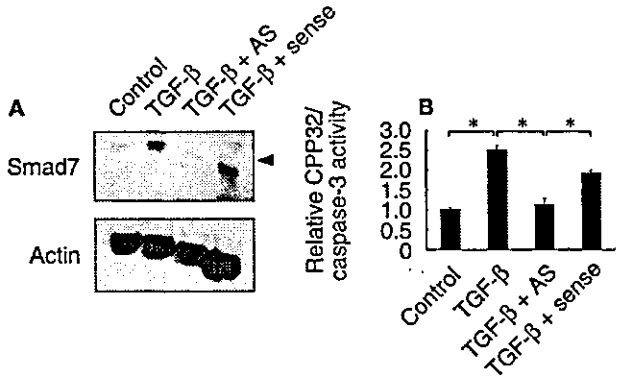

Fig. 6. TGF-β-induced apoptosis was forestalled by antisense oligonucleotide to Smad7 in CPP32/caspase-3 activity. (*A*) Western blot analysis of Smad7 on cell lysates from mesangial cells. Rat mesangial cells were exposed to TGF-β treatment (1 ng/mL), TGF-β treatment with antisense oligonucleotide (AS) to Smad7, or TGF-β treatment with sense oligonucleotide to Smad7 (sense) for 24 hours. Twenty micrograms of cell lysate protein was used for Western blot analysis. The protein production of Smad7 was determined by Western blot analysis using a specific antibody. (*B*) Rat mesangial cells were exposed to TGF-β treatment (1 ng/mL), TGF-β treatment with AS, or TGF-β treatment with sense for 24 hours. Cell lysate from adherent and floating cells was used for CPP32/caspase-3 assay (N = 6; data are mean ± SEM; $^*P < 0.001$).

Smad7 overexpression did not affect NF-κB activity in mesangial cells

Recent reports suggested that TGF-β and Smad7 induced apoptosis through different downstream pathways in MDCK cells [17] and podocytes [18]. These studies found that Smad7 inhibited the activity of the survival NF-κB, and proposed that Smad7-mediated apoptosis was induced by the inhibition of NF-κB as the cell survival factor in MDCK cells and podocytes. Thus, we tried to determine whether there was a relationship between Smad7 and NF-κB in mesangial cells. Rat mesangial cells were transiently transfected with NF-κB-promoter-luciferase-plasmid, and Smad7 and LacZ were overexpressed using the adenoviral method. As TNF-α is known to elicit significant expression of NF-κB [33], rat mesangial cells exposed to TNF-α were prepared as positive control for the NF-κB activation.

AdCMV-Smad7 inhibited the NF-κB–sensitive promoter-luciferase activity to about 76% compared with the AdCMV-LacZ control infection (Fig. 7A). However,

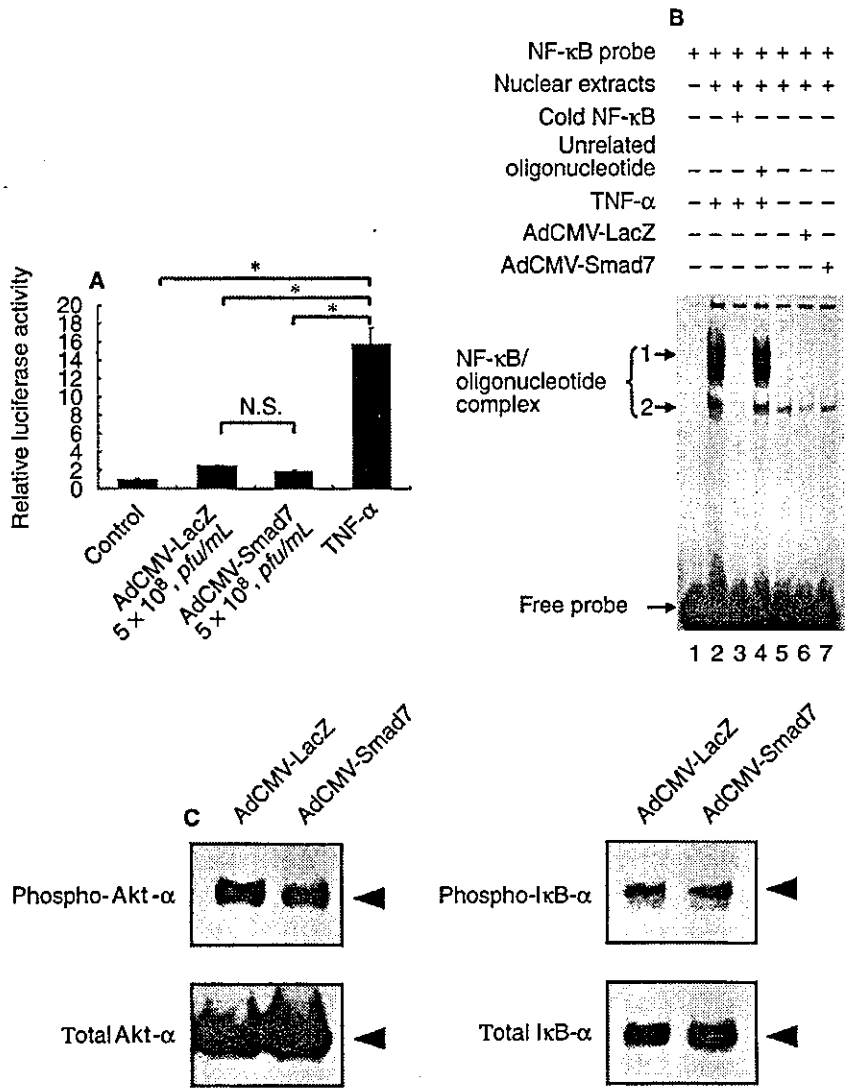

Fig. 7. Smad7 did not affect the nuclear factor-κB (NF-κB) activity in rat mesangial cells. (*A*) NF-κB-promoter-luciferase activity. Rat mesangial cells were transiently co-transfected with NF-κB-promoter-luciferase-plasmid and β-galactosidase plasmid, and then transfected with adenovirus (AdCMV-Smad7 or AdCMV-LacZ) or exposed to TNF-α (10 ng/mL). Luciferase-specific activity was determined by luciferase assay. The luciferase activity was normalized by β-galactosidase activity ($N = 6$; data are mean ± SEM; $^*P < 0.001$). (*B*) EMSA for detection of NF-κB DNA-protein complexes. Rat mesangial cells were transfected with adenovirus (AdCMV-Smad7 or AdCMV-LacZ) or exposed to TNF-α (10 ng/mL). Equal amounts of nuclear extracts (10 μg) were analyzed for NF-κB–specific DNA-protein binding using the ^{32}P-labeled consensus NF-κB probe. Negative control without nuclear extracts (lane 1) and positive control exposed to TNF-α (lane 2) and competition assay with 100-fold excess of unlabeled consensus, or unrelated oligonucleotides (lanes 3 and 4, respectively) were performed to assess the specificity of the reaction. The cells without any adenoviral transfection, the cells transfected with AdCMV-LacZ, and the cells transfected with AdCMV-Smad7 were performed (lanes 5, 6, and 7, respectively). NF-κB–specific protein-DNA complex (arrows 1 and 2) and free radiolabeled probe are indicated. (*C*) Western blot analysis on cell lysates from mesangial cells. Rat mesangial cells were transfected with either AdCMV-Smad7 or AdCMV-LacZ for 48 hours. Twenty micrograms of cell lysate protein was used for Western blot analysis. The protein production of Ser473-phospho-specific Akt, total-Akt, Ser32-phospho-specific IκB-α, and total IκB-α was determined by Western blot analysis using specific antibodies.

the inhibition was not significant. In addition, the NF-κB promoter-luciferase activities of cells transfected with AdCMV-Smad7 and AdCMV-LacZ were very low compared with the cells exposed to TNF-α.

We next examined the level of NF-κB in mesangial cell nuclear extracts by EMSA (Fig. 7B). After stimulation by 10 ng/mL TNF-α, extracts from positive control transfectants revealed DNA-protein complexes when incubated with the labeled consensus NK-κB probe. However, the NF-κB activation in the extracts from control cells and the cells transfected with AdCMV-Smad7 and AdCMV-LacZ was low and unchanged.

To examine the effect of Smad7 on Akt and IκB-α activities, cell lysates from mesangial cells transfected with AdCMV-Smad7 or AdCMV-LacZ were immunoblotted with antibodies directed against phospho-Akt and phospho-IκB-α, that is, the active forms of Akt and IκB-α. Figure 7C demonstrates that the protein levels of Akt phosphorylation observed in the cells transfected with AdCMV-Smad7 and AdCMV-LacZ were similar. Likewise, Smad7 had no noticeable effect on IκB-α activity.

These results suggested that Smad7 does not target NF-κB as a means of potentiating apoptosis in mesangial cells.

DISCUSSION

The present experiments using primary rat mesangial cells demonstrated, firstly, that Smad7 overexpression induced apoptosis, and secondly, that TGF-β–induced apoptosis was mediated by Smad7, but not by Smad2 or

Smad3. In addition, we showed that Smad7 overexpression increased the caspase-3 activity in the rat mesangial cells.

TGF-β has been reported to play a role in experimental glomerulonephritis and human glomerular diseases [10, 34, 35]. TGF-β expression was reported to be involved in the pathophysiology of mesangial cells in experimental nephritis [36]. Our current study demonstrates that TGF-β promoted apoptosis through caspase-3 activation in mesangial cells, and that Smad7 mediated TGF-β–induced apoptosis in the same cell type. Patel et al recently demonstrated that TGF-β promoted mesangial cell apoptosis partly through nitric oxide (NO) and a p53-dependent pathway, but they allowed that there could be a separate p53-independent pathway in TGF-β–induced apoptosis in mesangial cells [12]. Several pathways, including NO, p53, caspase cascade, and Smad7, may mediate TGF-β–induced mesangial cell apoptosis.

Smad7 was originally recognized as the auto-inhibitory downstream effector of TGF-β. While Smad7 has recently been reported to have an apoptotic effect on some cell lines [16–18], little is known about the apoptotic phenomena of TGF-β and Smad7 signaling in mesangial cells. A recent report by Schiffer et al revealed that podocytes underwent apoptosis in the early stages of progressive glomerulosclerosis in TGF-β transgenic mice [18]. Schiffer's group showed that this apoptosis was induced by TGF-β and Smad7, but they did not closely investigate the relationship between Smad7-induced apoptosis and mesangial cells [18]. Besides the podocytes, mesangial cells also are important in the pathogenesis of glomerular disease. Our study was the first, to our knowledge, to show that TGF-β and Smad7 induce apoptosis through caspase-3 activation in mesangial cells. Moreover, we demonstrated that R-Smads such as Smad2 and Smad3 do not influence the apoptotic change through caspase-3, and that overexpression of Smad7 potently induces apoptosis, while antisense oligonucleotides to Smad7 prevent the TGF-β-induced apoptosis. These findings provide evidence that Smad7 mediates most of the TGF-β–induced apoptosis that takes place in mesangial cells.

Our study also investigated the effect of Smad7 overexpression on NF-κB activity. NF-κB is an inducible transcription factor of well-established importance as an anti-apoptotic survival factor [33, 37–40]. We demonstrated, to our knowledge for the first time, that Smad7 overexpression does not affect the NF-κB activity in mesangial cells. This result suggests that the inhibition of NF-κB is not related to the pathway of Smad7-induced apoptosis in mesangial cells.

The ability of Smad7 to induce apoptosis is cell-type specific. Smad7 blocked TGF-β–induced apoptosis in hematopoietic cells [41, 42] and hepatocytes [15], whereas it only mediated TGF-β–induced apoptosis in prostatic carcinoma cells [16]. This difference could be a cell-specific discrepancy in the pathways of apoptosis and the pro-apoptotic effects of TGF-β and Smad7.

A previous report using podocytes suggested that TGF-β induced apoptosis via an activation of the classic effector caspase-3, while Smad7 induced apoptosis via an inhibition of NF-κB, independently of the caspase cascade [18]. Many different pathways of TGF-β– and Smad7-induced apoptosis in different cell types have been reported [12, 17, 18]. While there are no clear explanations for these different pathways, differences in cell types may be involved. Mesangial cells and podocytes both constitute the glomerulus, however, their characteristics are very distinct from one another. Mesangial cells have the potential to proliferate, while normal podocytes are terminally differentiated quiescent cells that may require the presence of p27 and p57 [43, 44]. Mesangial cells and podocytes in human kidney exhibit markedly different expression patterns of the cell cycle proteins [45]. Further, podocytes and mesangial cells differ in their cell injury response to diabetes and hyperlipidemia [46, 47]. In studies using TGF-β–transgenic mice [18], the two cell types exhibited different patterns of Smad7 expression and apoptosis over time.

To understand further the pathophysiology of glomerulonephritis, future studies should be initiated to resolve TGF-β– and Smad7-signaling networks and the functions of Smad7 in mesangial cells.

ACKNOWLEDGMENTS

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