

The Contribution of Increased Collagen Synthesis to Human Glomerulosclerosis: A Quantitative Analysis of $\alpha 2IV$ Collagen mRNA Expression by Competitive Polymerase Chain Reaction

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Summary

We previously reported that one of the main components of the sclerotic material in human glomerular diseases was type IV collagen. In this study we examined the contribution of increased synthesis to this process at the gene expression level. Sufficient material has not been available to study type IV collagen synthesis by normal or sclerotic glomeruli in humans. We took advantage of the availability of nephrectomy specimens from patients with renal carcinoma, and of the observation that approximately 50% of these patients develop varying degrees of glomerulosclerosis. We microdissected glomeruli from 10 patients and analyzed them using in situ reverse transcription coupled with polymerase chain reaction (PCR) analyses (in situ RT-PCR). $\alpha 2IV$ collagen mRNA, after reverse transcription into cDNA, was detected in all patients and appeared to be increased in those with glomerulosclerosis ($n = 5$). A competitive PCR assay was developed to quantitate this change. There was an average 3.7-fold increase in glomerular type IV collagen cDNA in patients with significant sclerosis. This change was not due to an increased number of glomerular cells. Thus, glomerulosclerosis in humans is associated with an elevation of glomerular type IV collagen gene expression, suggesting that increased synthesis of type IV collagen may represent one component of this process.

The rates of synthesis and degradation of glomerular basement membrane components in normal glomeruli and the role of aberrations in these processes in the development of glomerulosclerosis have been difficult to study in humans. We previously found, by immunofluorescence microscopy, that the accumulation of extracellular matrix in human glomerulosclerosis was largely composed of type IV collagen (1). Studies of the mechanism of this accumulation require direct examination of glomeruli, since glomerular changes have been shown to be regulated independently of other cortical elements (2, 3). The techniques currently available to study these changes at the glomerular level are either too insensitive in the adult, in the case of in situ hybridization in intact kidney (4), or require too many glomeruli in the case of Northern blots and ribonuclease protection assays. For these reasons, we developed a technique consisting of microdissection of human glomeruli from nephrectomy specimens, followed by in situ reverse transcription (RT)¹ of mRNA into

cDNA (5, 6), and detection of $\alpha 2IV$ collagen cDNA by PCR. A competitive PCR assay was developed to quantitate glomerulosclerosis-related changes in collagen type IV mRNA expression (7). This method of analysis allowed the reproducible measurement of the amount of $\alpha 2IV$ collagen cDNA derived from a fraction of single glomeruli.

Materials and Methods

Patient Population and Histology. Kidney tissue was obtained after patient consent from 10 individuals: nine underwent unilateral nephrectomy for diagnosis and/or treatment of a renal mass and one had an open renal biopsy for the nephrotic syndrome with severe renal insufficiency of unknown duration (see Table 1 for demographic and clinical data). One nephrectomy patient (WR1), previously diagnosed with von Hippel Lindau syndrome, had a benign tumor, but the rest had renal cell carcinoma. The patients had no history of diabetes mellitus, hypertension, or other systemic diseases associated with glomerular disease. Samples of cortical tissue distant from obvious tumor were placed in Carnoy's fixative, embedded in methacrylate or paraffin, and sections were stained with

¹ Abbreviations used in this paper: PAS, periodic acid-Schiff; RT, reverse transcription; VRC, vanadyl ribonucleoside complex.

periodic acid-Schiff (PAS). The presence of glomerulosclerosis, defined as an expansion of the mesangial matrix, was independently evaluated by histological examination of PAS-stained material and by immunofluorescence microscopy of frozen sections after exposure to an antibody to type IV collagen (PHM-12, Silenus, Westbury, NY).

Glomerular Microdissection. Several cortical fragments (~10 mm³), taken at a distance from the tumor, were placed at 4°C in a RNase inhibitor solution (vanadyl ribonucleoside complex [VRC]; Life Technologies, Inc., Gaithersburg, MD) as previously described (5, 6). The fragments were transferred to a microdissecting dish cooled at 4°C where glomeruli were separated from tubules in the same RNase inhibitor solution. After microdissection, isolated glomeruli were washed free of tissue debris and VRC in a second dish and transferred to a PCR tube containing a human placental RNase inhibitor (Boehringer Mannheim Corp., Indianapolis, IN). At least 50 glomeruli were isolated from each nephrectomy, and 20 from the one open renal biopsy.

Pooled Glomeruli and In Situ RT. Glomeruli were permeabilized immediately before RT with a mixture containing 0.9% Triton X-100, 1.2 U/μl of placental RNase inhibitor, and 5 mM dithiothreitol (5). Pools of five glomeruli were individually reverse transcribed with oligo dT, and 10 separate pools were combined after RT to lessen errors due to variations in glomerular size (8). RT was performed using a cDNA synthesis kit (Boehringer Mannheim Corp.) as previously described (5). Since the cDNA solution was found to be homogeneous in preliminary experiments, subsequent manipulations were performed using fractions of the cDNA solution prepared from pooled glomeruli (6).

RNA Extraction. The yield of cDNA from in situ RT samples was compared with that from RT of purified total RNA using 25 glomeruli microdissected from each of two patients. Total RNA was extracted using the RNazol method (Cinna/Biotech, Laboratories International Inc., Friendswood, TX). After RNA extraction, an aliquot corresponding to five glomeruli (in volume) was reverse transcribed and the resulting cDNA compared, after PCR amplification, with the cDNA prepared from in situ RT of 25 glomeruli isolated from the same patients.

Primers. The primers, 20–24 mer, were synthesized on a PCR-Mate DNA synthesizer (Applied Biosystems, Inc., Foster City, CA). The α2IV collagen primers were designed in the coding region of the NC1 domain in which unique nucleotide sequences allowed specific amplification of the α2 chain, but no other type IV collagen α chain. The size of the amplified product was 348 bp as predicted from the cDNA sequence (9). Its identity was confirmed by restriction enzyme analysis. Controls consisted of tubes in which the RT enzyme had been omitted, to assure lack of amplification of genomic DNA encoding α2IV collagen. These also served as PCR contamination controls.

Competitive PCR Assay. A quantitative method was established by developing a mutated cDNA template of α2IV collagen cDNA (7). A 69-bp deletion was created in the middle of the α2IV collagen cDNA molecule, resulting in a mutant cDNA of 279 bp. Competitive PCR was performed using the GeneAmp DNA Amplification kit (Perkin-Elmer Cetus Corp., Norwalk, CT) as previously described (6). Briefly, each PCR tube contained all the the amplification reagents and an amount of α2IV collagen cDNA template equivalent to that obtained from 1/10 of a glomerulus. Serial dilutions of the α2IV collagen-mutated cDNA were added and the reaction mixture was amplified (see Fig. 1). Duplicate quantitations of α2IV collagen cDNA were made on pools of 50 in situ RT glomeruli from each of the nine nephrectomies and on a pool of 20 glomeruli from the one renal biopsy. The range and

relative amounts of added mutant cDNA were determined in preliminary tests (7). The thermal cycler (Perkin-Elmer Cetus Corp.) was programmed so that the first incubation was performed at 94°C × 3 min. This was followed by 34 cycles consisting of the following sequential steps: 94°C × 1 min, 60°C × 1 min, and 72°C × 3 min. The final incubation was performed at 72°C × 7 min.

Analysis of PCR Products. The entire reaction mix was separated in a 4% Nusieve/Seakem (3:1) (FMC Bioproducts, Rockland, ME) agarose gel by electrophoresis. Photographs were taken with positive/negative 55 film (Polaroid Corp., Cambridge, MA) of DNA bands visualized with ethidium bromide staining and UV transillumination. The gel negative was scanned by laser densitometry (6) (Shimadzu Scientific Instruments Inc., Columbia, MD). The mutant/test cDNA ratio for each reaction tube was plotted as a function of the amount of mutant template and a straight line was drawn by linear regression analysis. The quantity of cDNA in the test sample was the amount at which the mutant/test band density ratio was equal to one (see Fig. 1) (6, 7).

Morphometry. The glomerular mean profile surface area was measured with a computer assisted planimeter in 63–101 glomeruli (mean = 90) using histological tissue sections (3). The number of glomerular cells was counted in 10–20 consecutively encountered glomerular profiles. The mean glomerular volume was calculated from the harmonic mean of the glomerular equatorial area, using the method of De Hoff and Rhines (10) for mean size of

Table 1. Patient Demographic and Clinical Data

Patient	Age	Sex	S _{cr} * mg/dl	Urinalysis	Tumor status
WR1	35	M	1.0	Normal	Benign
NIH4	49	M	1.2	11–20 RBC/HPF 1–5 WBC/HPF	RCCa Mets
WR3	52	M	1.0	Normal	RCCa
NIH1	50	M	1.3	11–20 RBC/HPF	RCCa Mets
WR2	75	M	0.9	3–4 RBC/HPF 1–2 WBC/HPF	RCCa
WR6	59	F	0.8	Normal	RCCa
NIH3	61	M	1.1	1–5 WBC/HPF	RCCa Mets
NIH2	39	F	0.8	Normal	RCCa Mets
WR5	61	F	1.1	Normal	RCCa
WR7†	23	M	4.0	4+ protein 3–5 RBC/HPF occ gran cast	-

* Abbreviations: S_{cr}, serum creatinine; RCCa, renal cell carcinoma; Mets, metastatic; WBC, white blood cells; HPF, high power field; occ gran cast, occasional granular cast.

† WR7 was a patient undergoing open local biopsy; all other patients underwent nephrectomy for a renal mass.

particles of similar shape. The relative glomerular cell number was determined for each patient using those with normal glomeruli as an arbitrary baseline value.

Statistical Analysis. The unpaired Student's *t* test, with the Welch approximation method, was used for comparisons between groups and a *P* value <0.05 was considered significant. All data were expressed as mean \pm SEM.

Results

Stability of $\alpha 2IV$ Collagen mRNA. Preliminary experiments revealed that the $\alpha 2IV$ collagen mRNA was stable for at least 150 min in glomeruli dissected from small fragments of cortex and kept at 4°C in the presence of VRC, a particulate RNase inhibitor (data not shown). Therefore, all subsequent RT reactions were performed within that time frame. The addition of a soluble, nonparticulate, RNase inhibitor (placental) to the tissue transport medium and the microdissection solution before RT did not improve the yield of cDNA recovery after RT.

In Situ RT vs Extraction of RNA. The relative amount of cDNA obtained from in situ RT of microdissected glomeruli to that obtained after extraction and RT of RNA from similarly microdissected glomeruli was compared in two

patients by standard and competitive PCR. Aliquots of cDNA from both preparations were amplified using $\alpha 2IV$ collagen primers. The density of the bands obtained after RT on glomeruli in situ was always greater than that after RT of purified RNA using standard PCR. The differences were quantitated by competitive PCR. The levels of $\alpha 2IV$ cDNA obtained from extracted RNA for the two patients were 15% and 50% of the values obtained with the in situ RT.

Variability of the RT Efficiency. The efficiency of RT was evaluated in both in situ reverse-transcribed glomeruli and extracted total glomerular RNA. The level of $\alpha 2IV$ collagen cDNA after in situ RT was assessed by competitive PCR in several separate samples of 25 glomeruli from each of three patients, two with glomerulosclerosis and one normal. The potential variability in RT efficiency was also evaluated in separate aliquots of glomerular RNA obtained from each of these patients. These experiments (data not shown) revealed an intersample variability of $\pm 20\%$, which approximates the 15% intraassay variation that we have found with competitive PCR. We concluded that the RT efficiency for a given mRNA template was very stable and that it was therefore valid to quantitatively compare in situ RT-PCR products between samples.

Glomerular Histology and Quantitation of $\alpha 2IV$ Collagen mRNA. Duplicate measurements of glomerular $\alpha 2IV$ collagen cDNA were performed in all patients (Table 2). These values were compared with the histological data. Of the nine patients who presented with a renal mass, four had diffuse glomerulosclerosis by light microscopy, characterized by moderate to severe expansion of the mesangial matrix. Pa-

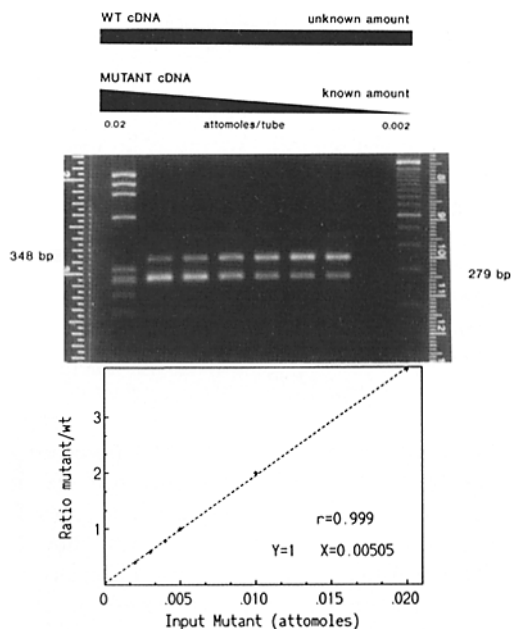


Figure 1. Competitive PCR quantitation of $\alpha 2IV$ collagen cDNA. The cDNA was from a pool of 50 microdissected glomeruli, isolated from a 61-yr-old male patient with glomerulosclerosis (NIH3). (Top) Reaction scheme and corresponding ethidium bromide-stained gel after PCR amplification. A constant amount of the wild-type test cDNA (*wt*), representing 1/10 of a glomerulus, and a decreasing amount of the mutant $\alpha 2IV$ collagen cDNA were added to a series of six tubes containing all PCR reagents. The mutant differed from the *wt* by the presence of a 69-bp deletion in the middle of the molecule. *Wt* and mutant bands were analyzed after 34 PCR cycles. (Bottom) Ratio of mutant/*wt* densitometric values vs the amount of mutant cDNA/tube. The amount of cDNA in the unknown is calculated from the equivalence point ($Y = 1$).

Table 2. Glomerular Histology, Morphometry, and $\alpha 2IV$ cDNA Levels

Patient	Sclerosis*	Relative cell number†	$\alpha 2IV$ collagen cDNA values‡
WR1	-	0.85	145 \pm 22
NIH4	-	0.90	174 \pm 30
WR3	-	1.13	85 \pm 15
NIH1	-	0.94	190 \pm 32
WR2	-	1.18	239 \pm 42
WR6	+	1.25	430 \pm 120
NIH3	+	1.35	548 \pm 41
NIH2	+	1.36	775 \pm 105
WR5	+	1.08	1046 \pm 74
WR7	+	0.22	323 \pm 10

* Each kidney specimen was examined for the presence of glomerulosclerosis on light microscopy. All tissue was embedded in methacrylate except for WR1 and WR7, which were paraffin embedded.

† The relative cell number of glomerular cells was determined by morphometric analysis as outlined in detail in Materials and Methods.

‡ Data from duplicate assays, expressed as mean \pm SEM in 10^{-4} attomoles/glomerulus.

tient WR7, who underwent open renal biopsy for the nephrotic syndrome with severe renal insufficiency, also had severe and diffuse glomerulosclerosis with a majority of the glomeruli being obsolescent. The finding of glomerulosclerosis did not correlate with patient age or the presence of clinically apparent metastatic disease.

Patients with severe glomerulosclerosis had a 2–6-fold increase in glomerular $\alpha 2IV$ collagen cDNA content (Table 2). The relationship between the presence of histological glomerulosclerosis and the type IV collagen cDNA levels is illustrated in Fig. 2 for two patients. Patient A, a 35-yr-old male with essentially normal glomeruli had $145 \pm 22 \times 10^{-4}$ attomoles/glomerulus in contrast to patient B, a 61-yr-old female with marked and diffuse glomerulosclerosis, who had $1046 \pm 74 \times 10^{-4}$ attomoles/glomerulus of $\alpha 2IV$ collagen cDNA. In the population of 10 patients, there was a significant increase in $\alpha 2IV$ collagen glomerular cDNA with glomerulosclerosis (624 ± 129 vs $167 \pm 26 \times 10^{-4}$ attomoles/glomerulus, $0.01 < p < 0.025$ for one-way analysis). The possibility that the increased mRNA level could reflect an increase in glomerular cell number in the corresponding patients was examined. The overall glomerular relative cell number varied from 0.85–1.36 (Table 2), except for the specimen WR7 in which the glomeruli were almost completely obsolescent. There was no significant increase in the relative number of cells per glomerulus in specimens with sclerosis when compared with normals (1.05 ± 0.21 vs 1.0 ± 0.07 , $p > 0.8$), suggesting that there was an increased amount of

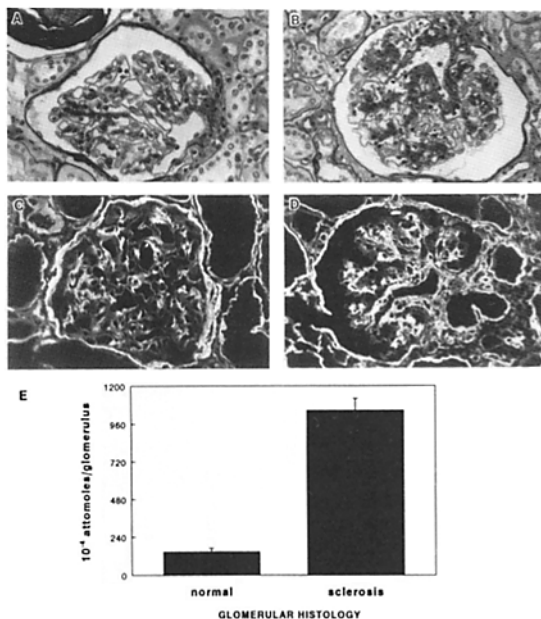


Figure 2. Changes in sclerotic glomeruli. (A–B) PAS-stained kidney sections from two unilateral nephrectomy specimens with renal carcinoma. (Left) Normal glomerular histology; (right) marked sclerosis. (C–D) Immunofluorescence microscopy, antibody to type IV collagen in the same kidneys. (E) Competitive PCR quantitation of $\alpha 2IV$ collagen cDNA in pools of 50 microdissected glomeruli from the same kidneys (values are 145 ± 22 vs $1046 \pm 74 \times 10^{-4}$ attomoles/glomerulus).

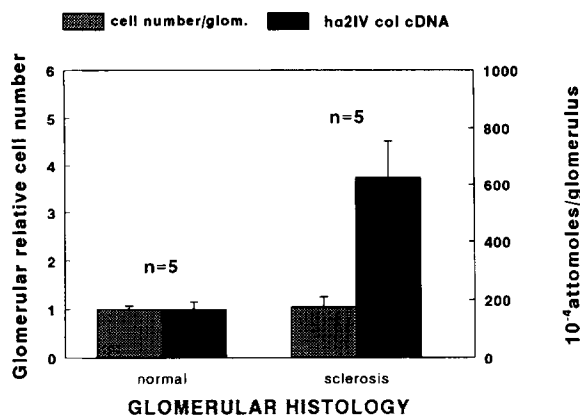


Figure 3. Relative cell number and $\alpha 2IV$ collagen cDNA levels. Five patients without glomerular sclerosis were compared with five patients with sclerosis. Difference between groups for the glomerular relative cell number was not significant ($p > 0.8$) whereas, for the $\alpha 2IV$ collagen cDNA levels, the difference was statistically significant ($0.01 < p < 0.025$).

type IV collagen cDNA on a per cell basis in the sclerotic glomeruli (Fig. 3). WR7 was a unique case in this analysis, since the glomeruli were almost completely obsolescent and contained only a small number of cells (Table 2). In this patient, normalization of the $\alpha 2IV$ collagen cDNA level to cell number resulted in a value similar to WR5, a patient with marked sclerosis and normal-sized glomeruli.

Discussion

The composition of the sclerotic matrix in humans and in animal models of glomerulosclerosis has been extensively studied by immunofluorescence microscopy (1, 11). A corresponding increase in the levels of extracellular matrix protein mRNAs has been demonstrated in isolated glomeruli from several animal models (12–14). Comparable studies in human patients have been hampered by the fact that classical molecular biology techniques lacked the sensitivity required to assess the levels of glomerular extracellular matrix gene expression in renal biopsies.

We studied glomeruli, microdissected from nephrectomy specimens, using a technique previously applied to mice (6) and rats (5). This method resulted in a glomerular preparation without tubular or vascular contaminants and allowed the isolation of essentially all the glomeruli available in a renal biopsy core. We took advantage of the availability of renal tissue in nephrectomies performed for renal carcinoma. In our population of nine nephrectomies, four of the patients had diffuse glomerulosclerosis which is very similar to the incidence of glomerulosclerosis reported in another series of 40 patients with renal carcinoma (15).

$\alpha 2IV$ collagen mRNA was detected in all patient glomeruli, normal and sclerotic, using standard RT-PCR. Since the levels appeared to be increased in cases with glomerulosclerosis, we used a competitive PCR assay to provide a more accurate assessment of the changes. $\alpha 2IV$ collagen cDNA was significantly elevated (3.7 times) in five patients with glomerulo-

sclerosis compared with five patients with normal glomeruli. The cDNA increase was not paralleled by an increase in glomerular cell number in the sclerotic glomeruli. To our knowledge, this is the first demonstration that collagen type IV mRNA is present in human glomeruli, and that the level is increased in adults with glomerulosclerosis, suggesting that these two events are related. It is somewhat surprising that the glomerular $\alpha 2IV$ collagen gene expression level was still high in a patient with extensive glomerular scarring (WR7). The persistence of a high level of expression suggested that sclerosis remained as an active process, even at late stages, a conclusion consistent with our recent observations in mice transgenic for bovine growth hormone (3). We found that glomerular cell turnover, in this mouse model, was high early in the development of sclerosis, and remained elevated in animals with advanced glomerulosclerosis and renal failure. These observations, in animals and in humans, raise the possibility that there may be a role for therapeutic intervention(s) at late stages of glomerular diseases.

The assessment of mRNA levels has most often been performed on extracted RNA, a method which results in variable losses during extraction and handling of small samples. For this reason, we chose to utilize *in situ* RT of mRNA in microdissected and permeabilized glomeruli (5). Comparison of these two methods of preparation, performed in our laboratory for several different molecules in mouse (6) and human glomeruli (E. P. Peten, unpublished observations), has always revealed a better yield of cDNA with *in situ* RT. Therefore, this method was used in the present study.

The competitive PCR technique has been shown to be applicable to small samples (6, 7). $\alpha 2IV$ collagen cDNA was reproducibly quantitated in as little as 1/10 of a glomerulus, in the range of 0.01–0.001 attomole. Although absolute values were obtained, the data apply only to the conditions reported herein, and it is to be expected that the values may change with further methodologic improvements.

One of the major problems in the quantitation of mRNA by competitive PCR has been the potential variation in the efficiency of RT. Several different schemes of competitive PCR utilizing a cRNA mutant, rather than a cDNA mutant, have

been recently described to address the problem of RT efficiency (16–18). The underlying assumption of this approach was that a synthetic exogenous cRNA would be reverse transcribed with the same efficiency as an endogenous mRNA, an unproven assumption (16). Moreover, competitive PCR with a cRNA mutant molecule requires quantitation of the total extracted RNA, a condition not attainable in microsamples of extracted glomerular RNA. Total glomerular cDNA also cannot be quantitated.

Another means to approach the question of RT efficiency is the parallel examination of a so-called housekeeping cRNA or cDNA molecule, the most commonly used molecules are GAPDH and β -actin. However, with the increased sensitivity afforded by competitive PCR, we (E. P. Peten, unpublished observations) and others (19, 20) have found that these mRNA molecules were expressed at widely varying levels in various human and animal disease states.

Finally, and most importantly, the data presented herein in humans, and previously in mice (6), and presented by others for rat RNA (21), represent strong evidence that both the *in vitro* and *in situ* RT efficiency is very stable for a given molecule. Therefore, as long as the stability of the RT efficiency is examined anew for each mRNA template, competitive PCR using a cDNA mutant is a reasonable method for quantitative comparisons for that mRNA species. This approach represents a substantial advantage for the prospective study of human renal biopsies.

In conclusion, we report the detection and quantitation of $\alpha 2IV$ collagen mRNA, after RT, in isolated glomeruli from a population of five patients with normal glomeruli and five patients with sclerotic glomeruli. There was a significant increase in glomerular $\alpha 2IV$ collagen cDNA content in patients with glomerulosclerosis. Since several different cDNAs, reflecting their glomerular mRNA content, could be quantitated by competitive PCR with as little as 10 microdissected glomeruli, this method can be applied to the study of percutaneous renal biopsies. This may represent a more sensitive way to evaluate prognosis and treatment efficacy in human renal diseases.

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