Abstract. The goal of this study was to improve a procedure for the extraction and storage of RNA from minute quantities of human renal tissue in clinical practice, using kidney biopsies and cadaveric donor kidneys unsuitable for transplantation. Collagen α1(IV) mRNA was analyzed as a measure for RNA integrity. The results show that at least 3 h may pass between microdissecting the renal tissue and the onset of cDNA synthesis without degradation of the glomerular mRNA. To extract the glomerular mRNA, microdissected glomeruli were incubated in a permeabilization solution. Treating glomeruli with collagenase IV before permeabilization had a deteriorating effect on the mRNA yield. The addition of reverse transcription mixture to the permeabilization solution in the presence of the glomeruli resulted in the highest cDNA yields. Storage of glomerular tissue in the presence of Nonidet P-40-based buffer for 1 wk at −70°C did not significantly affect the mRNA, but storage for 2 or 4 wk resulted in deterioration of the mRNA by approximately 40 and 95%, respectively. Furthermore, three methods for total RNA isolation from microdissected interstitial tissue were compared. An approximately 2.5 times higher yield of collagen α1(IV) mRNA was obtained with silica gel-based membrane spin technology than with a guanidine isothiocyanate/phenol chloroform or a lithium chloride/phenol chloroform method. Finally, this study shows for the first time reliable detection of collagen α1(IV) mRNA in biopsies that had been frozen for at least 10 yr at −70°C. These experiments have helped to improve a procedure for the processing of glomerular and interstitial tissue acquired from human kidney biopsies for mRNA analysis. This method is suitable for implementation in routine clinical practice.

The irreversible end stage in human chronic renal insufficiency is characterized morphologically by glomerulosclerosis and interstitial fibrosis. Accumulation of extracellular matrix (ECM) molecules is associated with the development of these lesions in the kidney. Previous experiments in animal models have shown that changes in mRNA levels of ECM molecules precede and predict morphologic alterations (1–4), thereby making it possible to prevent glomerulosclerosis by therapeutic intervention (5,6). These observations may herald a novel approach in the prediction and early treatment of human end-stage renal disease. Type IV collagen is one of the main components in both normal and diseased glomerular ECM (7–9). Both in humans and experimental animal models, the amount of type IV collagen mRNA is significantly higher in sclerotic glomeruli than in normal glomeruli (1,6,10,11). In various studies done in animal models, the initial mRNA steady-state levels of type I collagen (4) or type IV collagen (6) correlated with the relative rate of the development of glomerulosclerosis. In humans, only a few studies have been reported on glomerular mRNA levels of ECM molecules and fibrosis-regulating cytokines in microdissected biopsies (10,12–16).

Before investigating the clinical implications of ECM mRNA extraction in renal biopsies, it is necessary to optimize the steps that are involved in extracting the RNA from the tissue. In the investigation reported here, we improved a method for extraction and storage of minute quantities of RNA from glomerular and interstitial tissue, analyzing mRNA levels of the collagen α1(IV) molecule as a measure for RNA integrity. We show that we could detect collagen α1(IV) mRNA levels in biopsies that had been frozen up to 10 yr.

This method allows the quantification of mRNA levels for molecules involved in renal pathology, such as ECM molecules and cytokines, in clinically obtained human kidney biopsies.

Materials and Methods

Microdissection

To compare methods for glomerular and interstitial RNA extraction, three cadaveric donor kidneys, which were unsuitable for transplantation for technical reasons, were used (kidneys were obtained from Eurotransplant). We wanted to investigate whether microdissecting the renal tissue had an effect on the glomerular mRNA. Several biopsies from each donor kidney were taken with a 16-gauge needle. The cortical parts of the biopsies were transferred to a Petri dish
containing ice-cold phosphate-buffered saline (PBS) and cut into pieces approximately 2 mm long. The tissue was microdissected by two needles (Microance 0.6 × 25 mm; Becton Dickinson) using a stereo microscope (Zeiss, Oberkochen, Germany) to separate the glomeruli from the surrounding interstitial tissue. The glomeruli were transferred to a second dish containing ice-cold PBS and washed free of surrounding tissue debris. Eight batches of 10 glomeruli were put in tubes containing 40 U of RNase inhibitor (rRNasin) (Promega, Madison, WI), either directly or after 3 h incubation at 4°C in PBS, and then used for the RNA extraction experiments described below.

To investigate whether glomeruli were left in the interstitial fraction after microdissection, or, alternatively, whether interstitial components were carried over to the glomerular fraction, two pieces of cortical tissue 2 mm long were microdissected as described previously. Glomerular and interstitial tissues were separately pelleted by centrifugation, and fixed overnight in parafomaldehyde. After embedding in plastic, the tissues were completely cut up in sections and stained with toluidine blue for light microscopic analysis.

**RNA Extraction from Glomerular Tissue**

For these experiments, we used glomeruli obtained by microdissection of each of the three cadaveric donor kidneys, and compared several methods, described in the literature (10,12,15), suitable for RNA extraction from minute quantities of glomerular tissue. We used reverse transcription (RT)-PCR for amplification of the collagen α1(IV) mRNA present in the glomerular samples as a measure for RNA yield. Experiments were performed in duplicate.

From each kidney, eight batches of 10 glomeruli were pelleted by centrifugation for 1 min at 12,000 × g at room temperature to remove PBS. To investigate whether treatment of glomeruli with collagenase IV after microdissection but before permeabilization improves glomerular RNA extraction, four batches were incubated with 5 U of collagenase IV (Sigma, St. Louis, MO; catalog no. C1889) containing 40 U of rRNasin for 15 min at 37°C. As controls, four batches were incubated with PBS containing 40 U of rRNasin under the same conditions. The collagenase IV-treated glomeruli were washed once with 20 μl of PBS to remove the collagenase IV. We compared Nonidet P-40- and Triton X-100-based solutions for their ability to permeabilize glomerular cells. From each of the four batches, two were incubated in Nonidet P-40-based buffer (0.5% Nonidet P-40, 10 mM Tris buffer at pH 8.0, 10 mM NaCl, and 3 mM MgCl2, supplemented with 1 U/μl rRNasin) and two in Triton X-100-based solution (0.9% Triton X-100, 5 mM dithiothreitol, and 1 U/μl rRNasin) on ice for 5 min. The glomeruli were resuspended in a reaction volume of 10 μl by gently tapping the reaction tube.

For conversion of glomerular mRNA into cDNA, we used a mixture containing 5 U of avian myeloblastosis virus-reverse transcriptase (Boehringer Mannheim), 1 μM oligo(dT)15 (Boehringer Mannheim), 2 mM dNTP, and 10 U of rRNasin (Promega) in 1× RT buffer (Boehringer Mannheim). We compared two conditions of cDNA synthesis to find out which resulted in the highest cDNA yield: addition of RT mixture to the permeabilization solution either directly or after removal of the permeabilized glomeruli from this solution by centrifugation for 1 min at 12,000 × g at room temperature. The RT reactions were carried out for 1 h at 37°C and subsequently boiled for 5 min. Negative RT controls, using a solution containing all components except for the reverse transcriptase enzyme, were performed to check for contamination of the cDNA samples by genomic DNA.

**Storage of Glomerular Tissue**

To test the effect of storage time of glomerular tissue on the quality of the mRNA, eight batches containing 10 non-collagenase IV-treated glomeruli from each of the three donor kidneys were incubated either in 10 μl of Nonidet P-40 buffer, in 10 μl of Triton X-100 solution, both supplemented with 60 U of RNasin, or in 3 μl of rRNasin (60 U) alone, and were immediately frozen on CO2 ice. Under each condition, two batches (duplicates) were stored, either for 1 wk, 2 wk, or 4 wk, at −70°C. Two batches were frozen and immediately defrosted, and were used as controls. Afterward, the solutions containing the glomeruli were thawed on ice, and the glomerular cells were permeabilized in the solution they had been stored in. Nonidet P-40 buffer was used for glomerular batches that had been stored in rRNasin.

**RNA Extraction from Interstitial Tissue**

Six 16-gauge needle biopsies were taken from a cadaveric donor kidney. Cortical tissue samples approximately 2 mm long were microdissected as described above. Interstitial tissues were pelleted by centrifugation to remove the PBS and then weighed. Total RNA was isolated from the interstitial tissue either by silica gel-based membrane spin (SMGS) technology (RNeasy® mini kit; Qiagen, Chatsworth, CA), guanidine isothiocyanate/phenol chloroform (GITC/PC; TRizol® from Life Technologies BRL), or a lithium chloride/phenol chloroform (LiCl/PC) procedure. Weights of the tissues used for each of the three different protocols were 5.4 and 5.8 mg, 5.6 and 5.4 mg, and 7 and 6.8 mg, respectively. Interstitial tissues were resuspended in duplicate, in 350 μl of RNeasy® lysis buffer, 500 μl of TRizol® solution, or 500 μl of 3 M LiCl/6 M urea solution, respectively, and mixed vigorously (Ultra-Turrax; Janke & Kunkel, Zoetermeer, The Netherlands) for 45 s. The SGMS and GITC/PC methods were performed according to the supplier’s manual. The LiCl/PC method was performed according to an earlier report (17). For complete RNA precipitation with the Li/PC and the GITC/PC method, 5 μg of glycogen (Boehringer Mannheim) was added. The RNA obtained with the three RNA isolation methods was dissolved in equal amounts of RNase-free water (56 μl). Ten microliters of each RNA sample was incubated for 15 min at 60°C, and cDNA was synthesized by adding 10 μl of RT mixture. As a measure for RNA yield obtained with each of the three extraction methods, the collagen α1(IV) mRNA yield per milligram of tissue was measured by competitive RT-PCR.

**Storage of Nonmicrodissected Biopsy Tissue**

Biopsy specimens used in this experiment were obtained from patients with IgA nephropathy. Informed consent was given by the patients for use of part of the biopsy for scientific purposes. Biopsies were taken with an 18-gauge needle.

We used five biopsies that had been stored for different periods of time at −70°C (between 1 and 10 yr). After assessing the location of the cortex in each biopsy by light microscopy, a piece of cortical tissue 1 mm long was cut off. RNA was isolated with the SMGS method, and collagen α1(IV) cDNA was measured by RT-PCR in triplicate.

**PCR Conditions**

**Primers.** To distinguish PCR amplification of cDNA from that of genomic DNA by the size of the amplified products, human collagen α1(IV) primers (Life Technologies BRL) were localized to separate exons, spanning three introns. Collagen α1(IV) primer sequences were sense 5’-ATG TCA ATG GCA CCC ATC AC-3’ and antisense 5’-CTT CAA GGT GGA CCG CTG AG-3’, yielding an amplified product of 382 bp as predicted from the cDNA sequence. The primers were designed in the coding region of the NC1 domain of α1(IV) mRNA.
the collagen α1(IV) molecule, and are located approximately 300 bp upstream of the poly(A) tail.

**PCR Assay.** Glomerular collagen α1(IV) cDNA levels obtained in the experiments with the three cadaveric donor kidneys were semiquantitatively assessed by PCR. Each amplification reaction contained an amount of cDNA equivalent to that obtained from one-half of a glomerulus (i.e., 1 μl of the cDNA sample). The amplification mixture contained 1 μM of both primers, 0.2 mM dNTP, 2 mM Mg²⁺, 1 U of AmpliTaq, and 1× AmpliTaq buffer (Perkin-Elmer Cetus) in a total volume of 25 μl. The first incubation in the thermal cycler (Perkin-Elmer Cetus) was performed at 94°C for 7 min. This was followed by 35 cycles consisting of the following steps: 94°C for 1 min, 65°C for 0.5 min, and 72°C for 1.5 min. The final incubation was performed at 72°C for 7 min. All PCR assays were in exponential phase.

**Analysis of the PCR Products.** The amplified products were separated by agarose gel electrophoresis. Eight microliters of the reaction mixture was applied to a 1.5% agarose gel in Tris Borate/ethylenediaminetetra-acetic acid buffer. DNA bands were visualized by ethidium bromide staining and ultraviolet transillumination. The densitometric values of the amplified cDNA bands were calculated by densitometric image scanning (hardware used was The Imager from Molecular Dynamics; software was Appligene, Inc., version 2.03, and ImageQuant, version 4.2a).

**Statistical Analyses**

Data are presented as means ± SD. Paired t tests were used for statistical analysis of differences in PCR signals. P < 0.05 was considered statistically significant.

**Results**

**Microdissection**

With light microscopic inspection of sections obtained from both tissue fractions, no glomeruli were found in the interstitial sections, and no interstitial fragments were found in the glomerular sections.

We found that with 3 h between the onset of microdissection performed in ice-cold PBS and the start of cDNA synthesis, the relative glomerular collagen α1(IV) PCR signal was not significantly lower compared with glomerular tissue that was immediately processed for cDNA synthesis after microdissection (data not shown), indicating that the mRNA had not been degraded.

**RNA Extraction from Glomerular Tissue**

Figure 1 shows that the relative cDNA yield obtained from glomeruli treated with collagenase IV (0.16 ± 0.02 and 0.11 ± 0.05; bars 5 and 6) was significantly lower than that from nontreated glomeruli (1 ± 0.17 and 0.99 ± 0.04; bars 1 and 2; P < 0.01). Application of Nonidet P-40 or Triton X-100 solution for permeabilization of glomerular samples gives no significant difference in the efficiency of mRNA extraction. The relative amplified glomerular cDNA signals for Nonidet P-40- and Triton X-100-treated samples were 1 ± 0.17 (bar 1) and 0.99 ± 0.04 (bar 2; P = NS), respectively. Finally, Figure 1 shows that addition of RT mixture to the permeabilization solution in the presence of the glomerular cells results in a significantly higher collagen α1(IV) cDNA yield (1 ± 0.17 and 0.99 ± 0.04; bars 1 and 2) than with the glomerular cells removed (0.46 ± 0.15 and 0.45 ± 0.23; bars 3 and 4; P < 0.05).

**Storage of Glomerular Tissue**

Figure 2 shows that storage of glomeruli for 1 wk at −70°C in the presence of Triton X-100 solution (0.71 ± 0.06) or rRNasin alone (0.66 ± 0.17) resulted in a significant decrease of the collagen α1(IV) signal compared to glomeruli that had been frozen under the same conditions but immediately defrosted (1 ± 0.09 and 1 ± 0.17; P < 0.01). However, storage in the presence of Nonidet P-40 buffer for 1 wk did not result in a significant decrease (0.84 ± 0.21 versus 1 ± 0.13; P = NS). After 2 wk of storage in Nonidet P-40, Triton X-100, or rRNasin alone, relative levels had significantly decreased to 0.61 ± 0.19, 0.46 ± 0.22, and 0.37 ± 0.21, respectively (P < 0.05). After 4 wk, relative levels were 0.07 ± 0.05, 0.06 ± 0.04, and 0.05 ± 0.04, respectively (P < 0.01).

**RNA Extraction from Interstitial Tissue**

We found that the yield of interstitial collagen α1(IV) mRNA per milligram of tissue obtained with SGMS, measured
by assessment of the yield of the corresponding cDNA with the aid of competitive PCR, was at least 2.5 times higher than that obtained with GITC/PC or LiCl/PC (data not shown). Amplification of negative RT controls for all samples did not show any nonspecific bands, indicating that there was no contamination of genomic DNA.

Storage of Nonmicrodissected Biopsy Tissue

In previous experiments (unpublished), we found that it was not possible to obtain intact RNA from microdissected tissue that had been frozen and defrosted before microdissection. Therefore, mRNA levels in frozen biopsies could only be analyzed in nondissected, total cortical tissue. Collagen α1(IV) mRNA was measured with RT-PCR in biopsies that had been frozen for 1, 3, 5, or 10 yr at −70°C (Figure 3). Relative levels were 1 ± 0.16, 0.62 ± 0.17, 0.63 ± 0.12, and 0.81 ± 0.07, respectively. The mRNA did not significantly deteriorate between 1 and 10 yr of storage.

Discussion

In the present study, we compared different methods for extracting RNA from glomerular and interstitial tissue. We also investigated whether microdissecting or storing renal tissue for a certain period of time led to deterioration of the mRNA.

We analyzed mRNA levels for collagen α1(IV) by RT-PCR as a measure for efficiency of RNA extraction and for degradation of mRNA. One of our motives for looking at collagen α1(IV) is that it is a low abundance gene. When it is possible to detect such a transcript, it is likely that higher abundant transcripts certainly can be picked up. Another reason for our focus on collagen α1(IV) is that its mRNA has been shown to be upregulated in diseased and sclerotic renal tissue (1,6,10,11), suggesting a potential role in renal pathology.

We chose PCR primers that anneal to the collagen α1(IV) cDNA very near to the poly(A) tail of its corresponding mRNA, the starting site for the cDNA synthesis. In that way we sought to circumvent the inefficiency of the RT reaction and amplify as much cDNA as possible containing both primer sites. We believe that if one wished to perform an amplification using primers that are located more than 2 kb upstream of the poly(A) tail of any transcript, random hexamers should be used.
for the synthesis of cDNA. We specifically chose to amplify in all samples a collagen α1(IV) PCR product of one certain size. We doubt whether a comparison of intensity between this product and any longer products can be used as a test for integrity of the mRNA, because amplification efficiencies vary between PCR products of different sizes.

Our finding that microdissection for 3 h did not diminish the glomerular collagen α1(IV) mRNA yield corresponds with an earlier report that the mRNA for several collagen IV chains tested remained stable for even longer periods in dissected glomeruli kept at 4°C (12). When working with patient material, the time necessary for dissection of the biopsy lies within the time period of 3 h, hence ensuring that mRNA is not degraded.

In our study, treating glomeruli with collagenase IV before permeabilization resulted in a lower RNA extraction. This finding argues against the benefit of applying this pretreatment for improved RNA extraction from human glomeruli as described in an earlier report (15). We showed there was no significant difference in cDNA recovery between a Nonidet P-40-based and a Triton X-100-based permeabilization solution. However, when storing glomeruli for 1 wk at 70°C, the addition of the Nonidet P-40-based solution gave a better preservation of the mRNA than the Triton X-100-based solution, favoring the application of Nonidet P-40 in this protocol. After 1 mo of storage, the collagen α1(IV) mRNA signal had decreased by approximately 95%. This observation refines the finding in another study that glomerular mRNA levels for various collagen IV chains were reduced considerably after storage for 9 wk at −70°C (12). We conclude from our studies that it is preferable to perform cDNA synthesis on microdissected, frozen glomerular tissue within 1 wk of storage.

In previous studies, ECM mRNA quantification in relation to kidney disease has been focused mainly on glomerular and total cortical tissue. Recently, however, a study was reported in which RNA was extracted from both microdissected glomerular and interstitial tissue for the assessment of mRNA levels for ECM and ECM-regulating molecules (16). We sought to optimize the RNA isolation from microdissected interstitial tissue, and compared three RNA isolation methods with respect to their efficiency of RNA extraction. The higher RNA yield we obtained with the SGMS method, compared with the GITC/PC and LiCl/PC methods, may be explained by the fact that the SGMS method is based on the elution of bound RNA from a silica gel-based membrane. With the GITC/PC and LiCl/PC methods, phenol extraction and alcohol precipitation steps are required, which probably account for the lower RNA recovery. On the basis of these results, we recommend using the SGMS method for the isolation of RNA from small interstitial tissue specimens.

Finally, we showed that it is possible to detect collagen α1(IV) mRNA with RT-PCR in cortical RNA isolated from nonmicrodissected, frozen kidney biopsies that had been stored for at least 10 yr at −70°C. Microdissection of this tissue after defrosting results in degradation of the mRNA.

In conclusion, we have described an improved method for RNA extraction from both microdissected glomerular and interstitial tissue, and its potential for measurement of collagen α1(IV) mRNA in both tissues.

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