Fibrogenesis in progressive renal disease

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Chapter 2

Optimal method for RNA extraction from mouse glomeruli

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Abstract

Extraction of RNA has been described for rat and rabbit glomeruli but not for mouse glomeruli. Due to their small size, mouse glomeruli cannot be isolated by relatively simple sieving techniques. Based on recently reported methods for the isolation of mouse glomeruli, we developed an RNA isolation technique by performing comparative methodological studies. Two standard RNA extraction methods were compared. In addition in separate experiments the influence was studied of protease inhibitors and freezing and thawing of whole kidney prior to glomerular isolation, on the yield and degradation of RNA.

Therefore, kidneys were perfused with 10 ml 0.01 M PBS containing 1.25% $Fe_3 0_4$ through the aorta. Kidneys were decapsulated and passed through a 75 µm metal screen. After pelletting and washing, tubes were placed against a magnet and pelleted glomeruli were washed three times. In a second experiment, protease inhibitors were added to the PBS. As a third method, kidneys were frozen before the isolation of glomeruli. From isolated glomeruli, RNA was extracted using either cesium chloride or lithium chloride method.

The yields of RNA (OD 260) were highest using the lithium chloride method. Hybridization of Northern blots of extracted RNA with cDNA probes showed the best results when RNA was extracted using the lithium chloride method, while the cesium chloride method led to considerable degradation of RNA. Freezing of kidney tissue prior to RNA extraction led to the virtual absence of any signal. We then applied this method successfully in an *in vivo* model of experimental lupus nephritis.

This is the first description of an optimal protocol for the extraction of RNA from mouse glomeruli. From our studies we conclude that the lithium chloride method is superior for the extraction of RNA from mouse glomeruli. Adding of protease inhibitors during glomerular isolation is superfluous and freezing of kidney tissue prior to the isolation of glomeruli leads to total degradation of RNA.

Introduction

In the majority of kidney diseases, the production of a number of proteins is severely altered in renal tissue. Measurement of mRNA steady-state levels has become widely accepted as a method to obtain information about the biosynthesis of proteins in cell cultures and tissues, in addition to RNA translation rates and post-translational protein maturation. Northern blot or slot/dot blot techniques can be used to determine mRNA steady-state levels for individual molecules in RNA extracted from whole-kidney tissue or isolated glomeruli. Extraction of RNA has been described for rat and rabbit glomeruli, but not for mouse glomeruli (1,2). Since the size of mouse glomeruli is similar to that of mouse tubules, mouse glomeruli cannot be isolated by relatively simple sieving techniques, which implicated that, so far, only total mouse kidney tissues have been used for the extraction of RNA (3-5), which not gave information about mRNA levels in the glomeruli apart, or microdissection techniques were used to isolate very small amounts of glomeruli which give RNA just sufficient for a single competitive polymerase chain reaction experiment (PCR)(6).

However, the need for an effective method for the purification of RNA from mouse glomeruli exists, since mice are widely used for a number of experimental models for renal disease. The recent development of a rapid purification method for mouse glomeruli (7-9) has opened the way to mouse glomerular RNA extraction rendering amounts of RNA sufficient to perform a number of tests, including northern blots, spot/dot blots, PCR or competitive PCR experiments.

In this study we compared three modified methods for the isolation of glomeruli, to determine the optimal conditions for obtaining intact RNA. First, we applied a procedure for the isolation of glomeruli from fresh mouse kidney tissue described earlier (9). Second, protease inhibitors were used to find out whether it is necessary to use these very toxic chemicals to prevent degradation of the glomerular cells by autolytic enzymes during the procedure. Glomerular cells must survive the procedure, since in intact cells RNA is protected from RNase which degrades RNA. It is impossible to use specific RNase inhibitors such as sodium dodecyl sulfate (SDS) or diethyl pyrocarbonate (DEPC) because they lyse the cells, which would make it impossible to isolate the glomeruli. The third method called for the freezing of kidney tissue prior to the isolation of glomeruli. In addition, two RNA isolation methods were compared, i.e., cesium chloride (CsCl) extraction, and lithium chloride (LiCl) extraction. Lastly, we used kidneys from mice suffering from chronic graft-versus-host disease, an experimental model for lupus nephritis (10-12), to assess the influence of glomerular destruction and progressive glomerulosclerosis on the efficiency of isolation of glomeruli, and the extraction of RNA from those glomeruli, to determine the applicability of our method to experimental models of renal disease.

Subjects and methods

Animals

DBA/2 and C57BL10 mice were originally obtained from Olac Ltd. (Bicester, Oxfordshire, UK). (C57BL10xDBA/2)F1 hybrids were bred and kept in our own facilities. For these experiments, use was made of 60 male (C57BL10xDBA/2)F1 hybrid mice.

Experimental design

Glomeruli were isolated in three different ways. First, the kidneys of ten mice were perfused with phosphate-buffered saline (PBS) followed by perfusion with 10 ml PBS containing 1.25% Fe_3O_4 (Aldrich Chemie N.V./S.A., Brussels, Belgium)(9). Kidneys were decapsulated and stored in cold PBS. When all mice had been perfused, the kidneys were pressed through a 75 µm mesh metal screen (Twente Metaalgaas B.V., Hengelo, The Netherlands) with a flatted glass pestle, washed with a jet of PBS and collected in a siliconized metal dish. The suspension was allowed to settle for 20 min at 4°C in siliconized conical 50-ml centrifuge tubes. The supernatant was then removed and the pellet (5 ml) was resuspended in PBS. After the third run the tube was placed against a magnet (Dynal MPC™6, Dynal AS, Oslo, Norway) for 20 s before the supernatant was removed. Next, the tube was removed from the magnetic field, the inner wall was washed with PBS, and the resuspended glomeruli were washed by repeating this procedure three times, each with a 20-seconds magnetic collection phase. After the last wash the resuspended glomeruli were pelleted by centrifugation (30 s, 1200 g), and the pellet was snap-frozen in CO_2 ice, before being stored at -70°C until use. The total procedure for ten mice was performed within 1.5 hours.

In a second experiment, the same procedure was used, but all PBS contained a cocktail of protease inhibitors: 0.1% NaN_3 (Merck, Darmstadt, Germay), 0.5 mM phenylmethylsulfonyl fluoride (Sigma Chemical Company, St. Louis, MO, USA), 2 mM benzamidine-HCl, (Sigma) and 50 mM ε-amino caproic acid (Sigma).

For the third procedure, we used the same method just described except that the kidneys were snap-frozen in CO_2 ice-cooled isopentane prior to isolation of glomeruli. Kidneys were thawed in PBS without protease inhibitors.

Extraction of RNA

RNA was extracted in two ways after each of these 3 procedures for the isolation of glomeruli. In the first extraction procedure, the frozen pellet of glomeruli was resuspended in 3 ml guanidine isothiocyanate solution and mixed quickly (Ultra-Thorrax T25, Janke & Kunkel, IKAr-Labortechnick, Tamson, Zoetermeer, The Netherlands) for 1 min. Iron oxide and debris were

removed by centrifugation for 5 min at 1500 g. The supernatant was loaded onto 1.3 ml of a 5.7 M CsCl solution in an ultracentrifuge tube and centrifuged at 90,000 g for 16 hours in a Beckmann Sw50Ti rotor (13). After centrifugation, the supernatant was removed and the pellet resuspended in 1 ml RNase free TES (10 mM Tris, 5mM EDTA, 1 % SDS) and precipitated o/n with 1/10 volume 3 M Na-acetate and 2.5 volume ethanol at -20°C. The amount of RNA was determined by measuring of the optical density at 260 nm.

With the second method, RNA was extracted according to a lithium chloride procedure (14). Glomeruli were resuspended in a mixture containing 3 M LiCl and 6 M urea, and mixed quickly. After overnight incubation at 4°C, the solution was centrifuged for 1 hour at 12,000 g. The pellet was resuspended in TES, followed by two phenol/chloroform extractions and an ethanol precipitation.

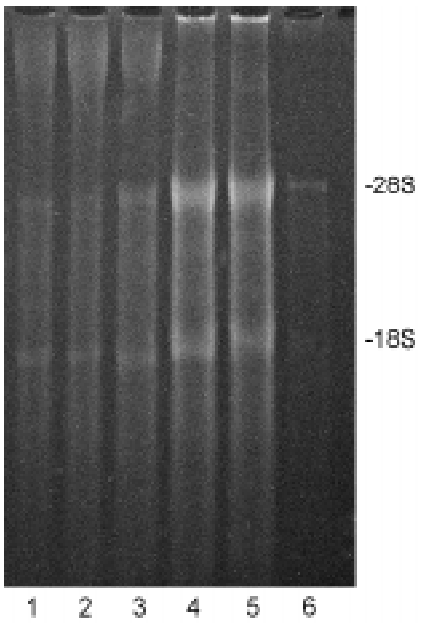

Figure 1. Ethidium bromide staining of the gel after electrophoresis. Lane 1 shows RNA extracted according to the CsCl method in combination with PBS. Lane 2 concerns RNA extracted according to the CsCl method in combination with protease inhibitors. Lane 3 refers to RNA extracted by the CsCl method after freezing of the kidneys. Lanes 4-6 represent the same glomeruli isolation methods as lanes 1-3 but in combination with LiCl RNA isolation method.

Northern blot analysis of RNA

The isolated RNA (20 µg/lane) was electrophoresed for 15 hours at 25V on a 1% agarose-formalin gel, stained with ethidium bromide to assess the quality of the RNA and blotted overnight to a Hybond™-C extra (Amersham, Little Chalfont, UK) membrane. After blotting, the membranes were rinsed with 3x SSC, air dried, and the RNA was baked on the membrane at 80°C for 4 hours.

cDNA probes and hybridization conditions

cDNA probes encoding for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or for collagen α1(IV) were labeled with ^{32}P with the use of a random primed labeling kit (Boerhinger Mannheim, Mannheim, Germany). The filters were pre-hybridized for 3 hours and hybridized o/n with the radio-(15)labeled probes at 65°C in 0.5 M $NaPO_4$ buffer (pH=7.0) containing 1 mM EDTA, 7% SDS, 1% bovine serum albumin and 50 µg/ml denatured salmon sperm DNA. The filters were washed twice with 2 x SSC and 0.1% SDS and twice with 0.2 x SSC and 1% SDS at 65°C. After being washed the filters were exposed to a Kodak XAR film (Eastman

Kodak, Rochester, New York, USA) at -70°C for 40 hours (GAPDH) or 10 days (Collagen α1(IV)).

Light microscopy.

For comparison of the percentages of sclerotic glomeruli in whole kidneys and in our preparation of glomeruli, the kidneys of a (C57BL10xDBA/2)F1 hybrid) mouse were perfused with iron oxide 12 weeks after the induction of chronic graft-versus-host disease as described in detail elsewhere(10). A sample of kidney tissue from this animal was fixed in 4% buffered formalin, embedded in paraffin, sectioned and stained with the Periodic acid-Schiff reaction for light-microscopical evaluation. Of the remaining renal tissue, the glomeruli were isolated, pelleted, and fixed in 1% glutaraldehyde and 4% formalin. The pellet was then embedded in Epon, sectioned, and stained with methylene blue for determination of the percentage of sclerotic glomeruli. For each of three tissue specimens at least 25 glomeruli were counted. Statistical analysis was performed with the unpaired Student's T-test.

Isolation method	OD260/280	Yield (μg)
CsCl - Method 1	>1.7	246
CsCl - Method 2	>1.7	266
CsCl - Method 3	>1.7	126
LiCl - Method 1	1.73	810
LiCl - Method 2	1.59	775
LiCl - Method 3	1.36	771

Table 1. Yields of RNA from mouse glomeruli using different methods. Method 1 is for RNA isolation from glomeruli isolated from fresh kidneys with the use of PBS. Method 2 is for RNA isolation from glomeruli isolated from fresh kidneys with the use of PBS containing protease inhibitors. Method 3 is used for RNA isolation from glomeruli isolated from frozen kidneys using PBS. (CsCl = cesium chloride RNA extraction, LiCl = lithium chloride RNA extraction).

Results

The glomerular magnetic suspensions contained no free fragments of tubuli, as was confirmed by light microscopy. Less then 10% of the glomeruli showed part of Bowman's capsule with a fragment of the proximal tubules attached. Yields of RNA are shown in Table I. RNA extraction according to the CsCl method rendered 200-300 μg RNA per twenty mouse kidneys either with or without protease inhibitors. The amounts of RNA isolated by the LiCl method were two to three times higher.

The quality of the RNA thus obtained was assessed by gel electrophoresis, as shown in Figure 1. RNA isolated according to the CsCl method showed two indistinct ribosomal bands (28S and 18S), and a smear in the top of the gel. LiCl isolation of RNA yielded two strong ribosomal bands. The 28S band was about twice as intense as the 18S ribosomal RNA band.

Hybridization of the membrane with a collagen α1(IV) cDNA probe gave a 6.8 kB band with the RNA isolated by the LiCl method from glomeruli isolated from fresh kidneys in PBS or in PBS containing the proteinase inhibitors (Figure 2, lanes 4 and 5). The other RNA preparations

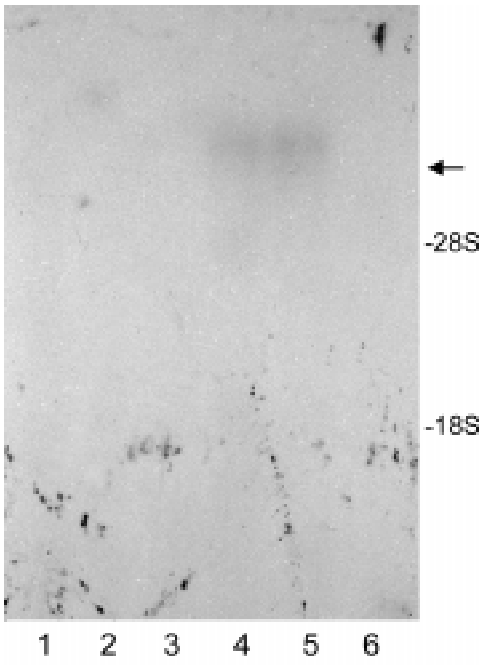

Figure 2. Hybridization of the filter with the collagen α1(IV) cDNA probe. Lane 1 represents RNA extracted by the use of the CsCl method in combination with PBS. Lane 2 refers to RNA extracted by the CsCl method in combination with protease inhibitors, lane 3 RNA extracted by the CsCl method after freezing of the kidneys. Lanes 4-6 represent the same glomeruli isolation methods as lanes 1-3 but in combination with the LiCl RNA isolation method.

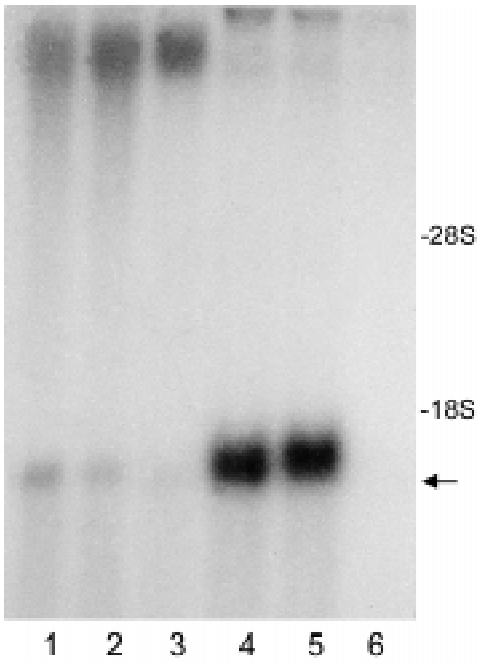

Figure 3. Hybridization of the filter with the GAPDH cDNA probe. Lane 1 represents RNA extracted by the CsCl method in combination with PBS, lane 2 RNA extracted by the CsCl method in combination with protease inhibitors, and lane 3 is RNA extracted by the CsCl method after freezing of the kidneys. Lanes 4-6 represent the same glomeruli isolation methods as lanes 1-3 but in combination with LiCl RNA isolation method.

failed to give a signal upon hybridization. Hybridization with a GAPDH cDNA probe resulted in an intense 1.3 kB band when the RNA was used that had been extracted according to the LiCl method in combination with the isolation of glomeruli from fresh kidneys with or without the use of proteinase inhibitors (Figure 3, lanes 4 and 5). The RNA extracted according to the CsCl method in combination with the isolation of glomeruli from fresh kidneys with or without proteinase inhibitors gave a weak 1.3 kB band and also a smear in the top of the membrane (Fig. 3, lanes 1 and 2). RNA extracted from glomeruli isolated from frozen kidneys did not give a visible signal.

Light-microscopical evaluation of the kidneys of animals with chronic graft-versus-host disease-related lupus nephritis showed focal and segmental glomerulosclerosis in 71.8% ± 4.1% (mean ± standard deviation of three counts, i.e., 25 glomeruli for each specimen) of the glomeruli.

In the isolated glomeruli preparation, 78.9% ± 7.6% of the glomeruli showed focal or segmental sclerosis. The difference between these two groups is not significant (p=0.225). Isolation of glomeruli from mice suffering from glomerulosclerosis gave the same or higher yields of glomeruli compared to isolation of glomeruli from normal mice. Moreover, the amounts of extracted total RNA for both groups were the same. Northern blots of RNA from these diseased mice showed intact RNA (16).

Discussion

In the present study we compared different methods for the isolation of glomeruli from mouse kidneys and different RNA isolation techniques to establish an optimal protocol for the extraction of RNA from mouse glomeruli. The time taken to isolate glomeruli was minimized which is crucial since ribonucleases may be released during the isolation procedure. Isolation of RNA from glomeruli according to the LiCl method yielded large amounts of RNA. Criteria for the intactness of this RNA were fulfilled: i.e., the ethidium bromide-stained gel showed no degradation products under the 18S ribosomal band, and the 28S band was about twice as intense as the 18S rRNA band. To evaluate the quality of the extracted RNA further, we performed hybridization with a cDNA probe coding for collagen a1(IV) mRNA. This probe is known to hybridize with a large mRNA transcript (6.8 kB) (17), that is relatively susceptible for degradation. Hybridization with this collagen a1(IV) cDNA probe yielded one distinct 6.8 kB band at the top of the blot just above the 28S ribosomal band, consistent with the localization of intact collagen α1(IV) mRNA (17). This indicates that no degradation of RNA had occurred during our extraction procedure, because degradation would have led to the presence of a smear under the 6.8 kB band of intact RNA. This also indicates, that during the approximate 1.5 h needed for glomeruli isolation the RNA is not degraded. This is probably due to the fact that the individual cells in the glomeruli stay intact, and that the use of protease inhibitors is not necessary for the isolation of intact RNA.

Extraction performed with the use of CsCl yielded smaller amounts of RNA. On a gel, the 28S and 18S ribosomal bands were not as prominent (Fig. 1, lanes 1 and 2), suggesting that part of the RNA had been degraded. This might explain why hybridization with the collagen α1(IV) probe did not give a signal and that with GAPDH only a weak signal (Fig. 3 lanes 1 and 2). The presence of a smear in the top of the gel and in the top of the blot after hybridization with the GAPDH cDNA probe might be due to contamination with DNA. With this CsCl extraction method too, no difference was found between glomeruli isolated with or without protease inhibitors.

Extraction of intact RNA from glomeruli isolated from frozen kidneys proved impossible. No signal was observed upon gel electrophoresis or after cDNA hybridization. Presumably, this

procedure leads to autolysis of glomerular cells and the release of RNase, which degraded the RNA during the glomeruli isolation. Therefore, the use of fresh kidney tissue is strongly advocated for the isolation of glomeruli and the subsequent extraction of glomerular RNA.

Extraction of poly A^+ mRNA, from isolated glomeruli, with the use of a commercially available kit (Fast track™ mRNA isolation kit, Version 2.1, Invitrogen corporation, San Diego, CA, USA) with oligo(dT) cellulose showed no signal after hybridization (data not shown) with the different probes. It might be expected that the iron particles used during the isolation of glomeruli might disturb mRNA isolation with this method because the iron particles were present throughout the isolation procedure. This in contrast with the other isolation methods in which they can be removed after centrifugation.

Finally, since the yield of RNA extraction may differ between normal and diseased kidneys, the influence of glomerular destruction on the efficiency of isolation of glomeruli was assessed to determine the applicability of our RNA extraction method to experimental models of renal disease. Interference with glomerular isolation due to the presence of glomerular sclerosis and capsule adhesion may lead to the extraction of a non-representative population of glomeruli and false mRNA steady-state levels, and should be excluded. To this end, we used end-stage kidneys from mice suffering from chronic graft-versus-host disease, an experimental model for lupus nephritis (10-12). Percentages of sclerosed glomeruli were similar in whole-kidneys and glomerular isolates, indicating that glomerular destruction does not affect our procedure for the determination of glomerular mRNA steady-state levels.

In sum, we have described here for the first time the optimal extraction procedure of RNA from glomeruli isolated from mouse kidneys. Based on our results, we advocate the use of fresh kidneys in combination with the LiCl RNA isolation method, which gave the highest yields and the best quality of RNA. We recently applied this method successfully in molecular biological studies on the development of glomerulosclerosis in experimental lupus nephritis in mice.

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