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Glomerular expression of nephrin is decreased in acquired human nephrotic syndrome

Peter N. Furness, Leon L. Hall, Jacqui A. Shaw and J. Howard Pringle

Department of Pathology, University of Leicester, Leicester, UK

Abstract

Background. Nephrin recently has been identified as a putative adhesion molecule, expressed in the glomerulus, in which mutations cause congenital nephrotic syndrome of Finnish type. We sought to determine whether expression of nephrin is altered in human glomeruli in patients with acquired nephrotic syndrome.

Methods. We performed PCR amplification of nephrin cDNA, using cDNA previously prepared from single human glomeruli plucked fresh from the surface of human renal biopsies. We had available four cases of nephrotic syndrome (one membranous, three minimal change) and six normal controls. PCR product quantitation was by gel densitometry, confirmed by enzyme-linked immunosorbent assay using a specific oligonucleotide probe. Results were corrected for reaction efficiency and glomerular cellularity by expression as a ratio to levels of the 'housekeeping gene' glyceraldehyde phosphate dehydrogenase.

Results. Glomerular levels of nephrin mRNA are significantly decreased in cases of minimal change nephrotic syndrome. An apparent reduction was also seen in the single case of membranous nephropathy which was available for study.

Conclusions. Abnormalities of nephrin expression appear to be associated with acquired as well as congenital causes of human nephrotic syndrome.

Key words: adhesion; glomerulus; nephrin; nephrotic syndrome; slit diaphragm

Introduction

The onset of nephrotic syndrome, of any cause, is accompanied by profound changes in the morphology of the glomerular podocytes which imply alterations in cell–cell and cell–matrix adhesion. Detachment from the glomerular basement membrane occurs [1,2], and the effacement of foot processes represents a simplification of architecture which demands massive remodelling of the podocyte intercellular junction, the slit diaphragm.

The molecular composition of the podocyte cell–matrix adhesion complexes has been largely determined in recent years, but the composition of the slit diaphragm remains surprisingly obscure. It contains the α-splice variant of ZO-1 [3], a protein which in the α+ form is a normal constituent of tight junctions. However, ultrastructurally and functionally, the slit diaphragm is not a tight junction. Other tight junction proteins such as cingulin [4] and occludin [5] are not present, and no other slit diaphragm-associated molecule has been identified.

The recent identification and sequencing of nephrin has, therefore, provoked considerable interest [6]. A mutation in the gene for this protein was found to be the underlying abnormality in the congenital nephrotic syndrome of Finnish type, where the glomerular podocytes fail to make normal foot processes or slit diaphragms. Nephrin mRNA is expressed in normal kidneys, and has been localized by *in situ* hybridization to the glomerular podocytes of a single human embryo [6]. Sequence analysis suggests that it is a novel transmembrane adhesion molecule of the immunoglobulin superfamily and, therefore, one might speculate that it could be a slit diaphragm component.

Since a mutation of this protein results in congenital nephrotic syndrome, it is reasonable to ask whether its expression is altered in acquired nephrotic syndrome.

We were able to address this question because we have for 2 years been accumulating a bank of cDNA transcribed from the mRNA of single human glomeruli, plucked from the surface of fresh diagnostic renal biopsies [7]. All cDNA is tested for genomic DNA contamination, and only samples known to be free of contamination are used. This cDNA appears to be stable. Review of our records revealed three cases of minimal change nephropathy and one case of 'early' membranous glomerulonephritis where suitable cDNA was available. We therefore investigated whether these cases showed altered expression of nephrin when compared with normal, non-proteinuric glomeruli.

Correspondence and offprint requests to: Dr P. N. Furness, Department of Pathology, Leicester General Hospital, Gwendolen Road, Leicester LE5 4PW, UK.

Materials and methods

Cases

The original diagnosis of idiopathic membranous glomerulonephritis or minimal change nephropathy was confirmed in each case by characteristic features on light microscopy, immunoperoxidase and electron microscopy, with appropriate clinical correlation. The case of membranous glomerulonephritis showed only minimal glomerular sclerosis (Ehrenreich and Churg grade 1–2 [8]) and had 13 g of proteinuria/24 h. The cases of minimal change nephropathy showed no glomerular abnormality other than foot process effacement, and had proteinuria of 13 g/24 h, 7 g/24 h and 13 g/24 h. Six control specimens were obtained from patients with asymptomatic microscopic haematuria, normal serum creatinine and normal biopsy findings by light microscopy, electron microscopy and immunocytochemistry for immunoglobulins and complement. In every control case, the conclusion after clinico-pathological discussion was that there was no evidence of renal disease to explain the microscopic haematuria. All patients were adult; age ranges 22–56 years (controls) and 19–72 years (nephrotic). Ethical committee approval and patient consent were obtained.

Extraction and reverse transcription of mRNA from single human glomeruli

Single glomeruli were plucked from fresh human renal biopsies by hand using fine forceps under direct vision using a dissection microscope. Glomeruli were obtained in <1 min after the biopsy had been taken, after the adequacy of the biopsy for diagnostic purposes had been confirmed. Glomeruli immediately were dropped into 100 µl of lysis/binding buffer [100 mM Tris–HCl, pH 8.0; 500 mM LiCl; 10 mM EDTA, pH 8.0; 1% (w/v) sodium dodecyl sulfate (SDS); 5 mM dithiothreitol (DTT)]. Glomerular mRNA was extracted and processed using oligo-(dT)-linked Dynabeads® (Dynal, Bromborough, UK), as described previously [7]. Glomeruli in lysis/binding buffer were incubated with 50 µg/ml proteinase K (Boehringer Mannheim) for 1 h at 37°C. The lysate was centrifuged for 30 s at 10 000 *g* and the supernatant mixed with oligo-(dT)-linked Dynabeads® (Dynal). The mRNA was allowed to anneal to the Dynabeads® for 10 min at room temperature. mRNA-linked Dynabeads® were washed twice in a buffer containing LiDS (Tris–HCl pH 8 10 mM, LiCl 0.15 M, EDTA 1 mM, LiDS, 0.1%; Dynal) and three times in the same buffer but without LiDS. Dynabeads® finally were resuspended in diethylpyrocarbonate (DEPC) water.

Dynabead®-linked mRNA was resuspended in reverse transcriptase buffer (Promega) containing 1 mM DEPC-treated dNTPs (Pharmacia Biotech), 25 U RNAsin (Promega) and 5 U of AMV reverse transcriptase (Promega). Priming was by the oligo-(dT) Dynabeads®, and incubation was for 1 h at 42°C. The resultant cDNA was stored at 4°C.

PCR and measurement of cDNA

PCR primers were designed using the software package GCG Prime (Genetics Computer Group, Madison, WI) on sequences obtained from the EMBL database to be specific for the nephrin gene. Primers for glyceraldehyde phosphate dehydrogenase (GAPDH), a 'housekeeping gene', were used to control for sample cellularity and extraction efficiency. GAPDH has been used widely as a 'housekeeping gene' and we previously have reported its use in the glomerulus in this context [7,9]. Sequences were as follows:

Nephrin
3′ (reverse) TAC ACC AGA TGT CCC CTC AG,
5′ (forward) TCT TAT TCC CGA GGT TTC AC;

GAPDH
3′ (reverse) AGA ACA TCA TCC CTG CCT C,
5′ (forward) GCC AAA TTC GTT GTC ATA CC.

These sequences were synthesized by Gibco–BRL. Forward primers were biotinylated at source.

cDNA-linked beads were washed once in Tris-EDTA and resuspended in PCR buffer [Tris, pH 8.8, 45 mM; $(NH_4)_2SO_4$, 11 mM; $MgCl_2$, 4.5 mM; dNTPs, 200 µM; ultrapure bovine serum albumin (BSA; Advanced Protein Products Ltd), 110 µg/ml; β-mercaptoethanol, 6.7 mM; EDTA, pH 8, 4.4 µM] containing 10 pmol of forward primer and 10 pmol of reverse primer.

PCR product was quantified using agarose electrophoresis, visualization using ethidium bromide and UV light, and densitometry using the gel analysis macro supplied with NIH Image (available free from http://rsb.info.nih.gov/nih-image/).

The PCR products were not sequenced, but the specificity of the amplification was confirmed by parallel measurement using an enzyme-linked immunosorbent assay (ELISA) method [9], which involves detecting the product with the following specific probes:

nephrin
GTC CCA TCT GCA CTT CAT CG;

GAPDH
GTT GAA GTC AGA GGA GAC C.

CovaLink plates (Life Technologies Ltd.) were biotinylated by incubation overnight with *n*-hydroxysuccinyl biotin [20 µg/ml in phosphate-buffered saline (PBS)] at room temperature. Plates were then washed three times with 'buffer 1' [2 M NaCl; 40 mM $MgSO_4$; 0.05% (v/v) Tween-20 in PBS] and treated with avidin (50 µg/ml in buffer 1) for 30 min at room temperature, with agitation. Following three washes in 'buffer 2' (0.02% Tween-20 in PBS), plates were treated with 3% PBS/BSA for 15 min at room temperature, with agitation. PCR products were dissolved 1 : 100 in PBS/BSA and allowed to bind to the avidin-coated plates for 1 h at room temperature, with agitation. Unbiotinylated (reverse strand) PCR products were denatured from the biotinylated (forward strand) by addition of an equal volume of 0.25 M NaOH for 10 min at room temperature. Plates were then incubated for 1.5 h at 42°C with the gene-specific oligonucleotide probe [previously labelled with a 1 : 5 ratio digoxigenin-11-dUTP : dATP by terminal deoxynucleotidyl transferase (Promega, Southampton, UK) in the supplied buffer and diluted to 0.2 pmol/100 µl rapid hybridization buffer (Amersham International, Amersham, UK)]. Plates were then washed three times in 'buffer 2' before incubation with alkaline phosphatase-conjugated anti-digoxigenin diluted 1 : 500 in PBS/BSA, for 30 min at room temperature, with agitation. Plates were washed three times in 'buffer 2' followed by incubation in *p*-nitrophenyl phosphate 1 mg/ml in 1 M diethanolamine pH 9.8, for 1.5 h at 37°C. Samples were then read at 405 nm with a differential of 630 nm on a multi-well plate reader (Denley Instruments Ltd., Billingshurst, UK).

All ELISA measurements were performed in duplicate with appropriate controls for non-specific probe binding and plate quality.

Statistical analysis was by ANOVA (Microsoft Excel), confirmed by Mann–Whitney U test (MiniTab) as with the small sample numbers a normal distribution could not be proven.

Results

PCR amplification of GAPDH and nephrin cDNA was achieved in each case. When corrected for GAPDH expression, there was a consistent decrease in glomerular expression in the nephrotic cases (Figure 1 and Table 1; $P=0.014$ by Mann–Whitney). If the single case of membranous glomerulonephritis was excluded, the difference between the cases of minimal change nephropathy and normal remained statistically significant ($P=0.028$ by Mann–Whitney). These densitometric results were confirmed by the ELISA measurements ($P=0.02$ and 0.043 respectively).

Discussion

We have confirmed that nephrin mRNA is produced in the normal human glomerulus, and demonstrated for the first time its expression at that site in the adult. We acknowledge that we have relatively few cases available for study, and local circumstances make it impossible for us to recruit more in the near future.

Table 1. Ratio of expression of mRNA for nephrin : GAPDH, as measured by densitometry and ELISA

Cases	Diagnosis	Densitometry	ELISA
1	Normal	1.65	0.60
2	Normal	1.60	0.94
3	Normal	2.65	1.67
4	Normal	1.97	1.27
5	Normal	2.55	1.41
6	Normal	1.74	1.01
7	Minimal change	0.41	0.29
8	Minimal change	0.71	0.34
9	Minimal change	0.36	0.30
10	Membranous	1.54	0.97

P-values given in the text.

However, despite the small numbers, we have found a statistically convincing proportionate decrease in the levels of nephrin mRNA in human glomeruli from patients with nephrotic syndrome, specifically in minimal change nephropathy.

Measurement of mRNA levels by RT–PCR has been shown to be able to detect relatively small changes in gene expression in single animal glomeruli [10], and we recently have demonstrated excellent reproducibility of our methods when applied to single human glomeruli [9], with standard deviation $\sim 10\%$ of the mean value. We therefore argue that in the present context, these methods are reliable.

Our results indicate that changes in nephrin expression in acquired human nephrotic syndrome parallel the pathophysiology of congenital nephrotic syndrome of Finnish type. This condition is inherited in a recessive manner; heterozygotes do not have proteinuria. However, the level of nephrin expression in heterozygote glomeruli has not yet been measured and, therefore, it cannot be assumed to be decreased.

It is possible that the difference which we have detected is constitutional rather than acquired, and that such a constitutional decrease predisposes to the development of nephrotic disease; however, we think this unlikely. We assume that the lower level of nephrin mRNA is acquired as part of the development of the disease. It will not be difficult to clarify this with future human and animal studies. Either interpretation argues that a deficiency of nephrin is a general feature of heavy glomerular proteinuria, rather than being a specific defect confined to the inherited syndrome.

Heavy proteinuria of any cause is accompanied by ultrastructural changes which imply alterations in podocyte adhesion, both to the basement membrane and between podocytes. This is demonstrated by detachment of podocytes [11] and foot process effacement [12], respectively. The cell–matrix adhesion of the podocyte is an integrin-containing focal contact, a type found throughout the body. The slit diaphragm, in contrast, is a structure which is confined to the kidney. The expression of nephrin and the primary abnormalities of Finnish type nephrotic syndrome are also confined to the kidney [6]. The sequence of nephrin suggests a role as an adhesion molecule.

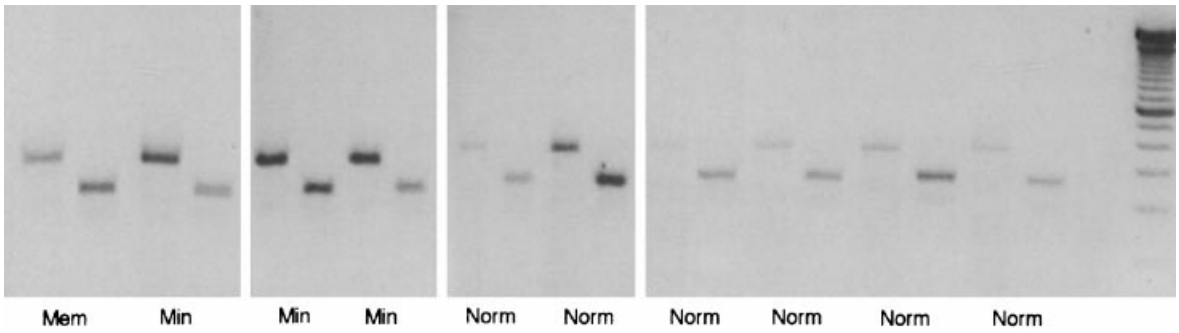

Fig. 1. Electrophoresis of PCR products from single glomeruli. Mem = membranous glomerulonephritis; Min = minimal change nephropathy; Norm = normal. In each case, the lane on the left represents amplification of GAPDH cDNA, that on the right (lower bands) represents nephrin.

Consequently, it is tempting to suggest that nephrin may be involved in the glomerular slit diaphragm, and that its decreased expression in nephrotic syndrome is related to the changes seen in that structure in heavy proteinuria.

The molecular composition of the slit diaphragm is poorly understood. The only protein which it is known to contain is the α-splice variant of the tight junction protein ZO-1 [3]. This splice variant has been associated with junctions which have greater permeability than conventional tight junctions. Other tight junction proteins are not present [4,5,13]. Using a careful quantitative immunocytochemical approach in human nephrotic syndrome, we were unable to detect changes in levels of ZO-1 or the other major glomerular adhesion proteins, though we did find a dramatic increase in phosphotyrosine [13]. In a rat model of nephrotic syndrome, changes in phosphorylation of glomerular ZO-1 have been reported [14], and in other locations this has been associated with increased junctional permeability [15]. The location of ZO-1 has been shown by immunoelectron microscopy to change in nephrotic syndrome [16].

The identification of nephrin as another molecule intimately involved in the control of glomerular permeability provides new avenues for investigation of this important subject.

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