In Vivo Expression of Putative *LMX1B* Targets in Nail-Patella Syndrome Kidneys

Laurence Heidet,* Ernie M. H. F. Bongers,[†]
Mireille Sich,* Shao-Yu Zhang,* Chantal Loirat,[‡]
Alain Meyrier,[§] Michel Broyer,[¶]
Gérard Landthaler,^{||} Bernadette Faller,**
Yoshikazu Sado,^{††} Nine V. A. M. Knoers,[†] and
Marie-Claire Gubler*

From INSERM U574* and the Département de Néphrologie Pédiatrique,[¶] Université René Descartes, Hôpital Necker-Enfants Malades, Paris, France; the Service de Néphrologie,[‡] Hôpital Robert Debré, Paris, France; the Département de Néphrologie,[§] Hôpital Européen Georges Pompidou, Paris, France; the Service de Pédiatrie,^{||} Hôpital Charles Nicolle, Rouen, France; the Service de Néphrologie,^{**} Hospices Civils, Colmar, France; the Department of Human Genetics,[†] University Medical Centre, Nijmegen, The Netherlands; and the Division of Immunology,^{††} Shigei Medical Research Institute, Okayama, Japan

The nail-patella syndrome (NPS) is characterized by nail and bone abnormalities, associated with glomerular involvement in ~40% of patients. Typical glomerular changes consist of fibrillar material in the irregularly thickened glomerular basement membrane. NPS is inherited as an autosomal dominant trait and caused by heterozygous loss of function mutations in *LMX1B*, a member of the LIM homeodomain protein family. Mice with homozygous inactivation of the gene exhibit nail and skeletal defects, similar to those observed in patients, associated with glomerular abnormalities. Strong reduction in the glomerular expression of the $\alpha 3$ and $\alpha 4$ chains of type IV collagen, and of podocin and CD2AP, two podocyte proteins critical for glomerular function, has been observed in *Lmx1b* null mice. The expression of these proteins appeared to be regulated by *Lmx1b*. To determine whether these changes in podocyte gene expression are involved in the development of NPS nephropathy, using immunohistological techniques, we analyzed the podocyte phenotype and the renal distribution of type IV collagen chains in the kidneys of seven NPS patients with severe glomerular disease. We also examined the nature of the fibrillar material present within the glomerular extracellular matrix. The glomerular basement membrane fibrillar material was specifically labeled with anti-type III collagen antibodies, suggesting a possible regulation of type III collagen expression by *LMX1B*. The expression of the $\alpha 3$ and $\alpha 4$ chains of type IV collagen, and of podocin and CD2AP, was found to be normal in the seven patients.

These findings indicate that heterozygous mutations of *LMX1B* do not appear to dramatically affect the expression of type IV collagen chains, podocin, or CD2AP in NPS patients. (Am J Pathol 2003, 163:145–155)

The nail-patella syndrome (NPS) (MIM 161200) is an autosomal dominant disease linked to chromosome 9q34. It is characterized by nail and bone abnormalities. Since its first description by Little in 1897,¹ more than 500 cases have been reported. Nail dysplasia, hypoplastic or absent patellas, elbow dysplasia, and iliac horns are highly penetrant features of the disease. They are observed in 75 to 90% of patients.^{2,3} Other skeletal defects are less frequent. Open-angle glaucoma has recently been recognized as an additional feature of the disorder.⁴ Glomerular involvement, which affects ~30 to 40% of patients, was described later.^{5–7} Persistent and moderate proteinuria, possibly associated with microscopic hematuria is the revealing symptom. Progression to nephrotic syndrome and/or end-stage renal disease occurs in ~10% of cases. The prognosis of NPS depends on the severity of the glomerulopathy, the course of which is unpredictable, even among members of the same family.² The ultrastructural changes of the glomerular basement membrane (GBM) in the NPS are characteristic. They consist of irregular GBM thickening with electron-lucent areas containing bundles of a fibrillar material that has the periodicity of interstitial collagen.^{8,9} Bundles of fibrils are also present in the mesangial matrix. The lesions are observed in proteinuric but also, surprisingly, in nonproteinuric NPS patients.^{10–12} Based on these renal changes, it had been initially suggested that the primary defect in NPS could affect one of the basement membrane components. The pathogenic role of fibrin has also been discussed on the finding of glomerular fibrin deposits in a spontaneously aborted 18-week fetus whose mother had NPS.¹³

Two groups found *LMX1B* mutations associated with NPS.^{14–16} *LMX1B* is a LIM-homeodomain transcription factor playing a key role in limb development.¹⁷ It estab-

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Address reprint requests to Dr. Marie Claire Gubler, INSERM U574, Tour Lavoisier 6^{ème} 2^{ème} étage, Hôpital Necker Enfants Malades 149, rue de Sèvres, 75743 Paris, Cedex 15, France. E-mail: gubler@necker.fr.

Table 1. Clinical and Morphological Features in the Seven NPS Patients

Patients	Sex	Age	H	Status at the time of kidney examination							EM GBM lesions	Renal status at last follow-up
				Pu/NS	Renal function	Nail dysplasia	Patellae	Iliac horns	Elbow dysplasia	LM		
1	F	5.6	—	1g/24 hours	N	+	Absence	+	+	MGC	+	Pu 13
2	M	13.6	+	<0.5 g/24 hours	N	+	Hypoplasia	+	+	MGC	+	Pu 26
3	M	16	+	NS	ESRD	+	Hypoplasia	+	—	ESK	ND	ESRD 16
4	M	1.6	—	NS	N	+	+	+	+	MGC	—	
		5	—	NS	N	+	+	+	+	FSGS	+	ESRD 17
5	F	19	—	NS	CRF	+	Absence	+	+	FSGS	+	ESRD 20
6	F	7	—	NS	ESRD	+	Absence	+	+	ESK	+	ESRD 7
7	M	25	—	NS	N	+	Hypoplasia	+	+	FSGS	+	ESRD 27

H, hematuria; Pu/NS, proteinuria/nephrotic syndrome; N, normal; LM, light microscopy; EM, electron microscopy; GBM, glomerular basement membrane; ESRD, end-stage renal disease; CRF, chronic renal failure; MGC, minimal glomerular changes; ESK, end-stage kidney; FSGS, focal segmental glomerular sclerosis.

lishes dorso-ventral patterning of the limb where it is expressed in a temporally and spatially restricted manner in the mouse and the chicken.^{15,18,19} Mice with homozygous inactivation of the gene die within 24 hours after birth. They exhibit nail and skeletal defects similar to those observed in the human cases, defects that are associated with renal abnormalities.^{15,20–22} More than 90 *LMX1B* mutations have now been reported in NPS patients.^{23–27} They are located in the LIM or homeodomain regions and are thought to result in a loss-of-function of the mutated protein.^{24,28} The role of *LMX1B* in limb patterning provides a satisfactory explanation for the occurrence of nail and skeletal anomalies in NPS patients.

The link between *LMX1B* mutation and GBM alteration was less easy to understand. However, recent data resulting from the analysis of the expression and the role of *Lmx1b* in the mouse kidney shed light on the mechanisms leading to GBM abnormalities: the gene is only expressed in podocytes from the S-shaped body stage onward and persists in postnatal mature podocytes.^{15,20} Moreover, genes expressed by podocytes were recently shown to be down-regulated in *Lmx1b*-null mice. Specifically, a reduced expression of *Col4a3* and *Col4a4* transcripts, with a stronger effect on *Col4a4*, has been observed in *Lmx1b* null mice, associated with diminished expression of the $\alpha 3$ and $\alpha 4$ chains of type IV collagen in the GBM.²⁰ These two chains are known to be important for long-term glomerular filtration as mutations in *COL4A3* or *COL4A4* cause Alport syndrome, a progressive hereditary nephritis in humans.^{29,30} *LMX1B* was shown to bind to a putative enhancer in both human and mouse *COL4A4* intron 1.²⁰ On the other hand, the expression of podocin that is mutated in a subset of children with steroid-resistant nephrotic syndrome,³¹ was found to be severely reduced in *Lmx1b* null mice, and potentially regulated by *LMX1B*.^{21,22} The expression of CD2AP, a podocyte protein also required for normal glomerular function,³² was also greatly reduced in *Lmx1b* null mice.²² Globally these findings indicate that *LMX1B* regulates the expression of several podocyte genes critical for podocyte differentiation and function. They strongly suggest that defects in GBM type IV collagen and changes in podocyte phenotype could contribute to the glomerular disease in NPS. However, whereas in hu-

mans, the disease is associated with heterozygous *LMX1B* mutations thought to result in haplo insufficiency, no renal symptoms, and no morphological abnormalities were found in *Lmx1b*+/- mice, even after 1 year of life.²¹

To determine whether these changes in podocyte gene expression are involved in the development of glomerular lesions in NPS, we analyzed the podocyte phenotype and the renal distribution of type IV collagen chains in the kidneys of NPS patients, most of them suffering from severe glomerular disease. In parallel, using light and ultrastructural immunohistology, we examined the nature of the fibrillar material present within the glomerular extracellular matrix.

Patients and Methods

Patients and Kidney Tissue Specimens (Table 1)

Renal tissue from seven patients (six biopsies and two nephrectomies performed at the time of transplantation) were available for this study. All patients had a typical association of nail dysplasia, abnormal or absent patellae, elbow dysplasia, and iliac horns. The diagnosis was made at birth in patients 4 and 6 because of skeletal abnormalities with pseudo-arthrogryposis. A family history for NPS was found in four patients (patients 1, 2, 3, and 7) including monozygotic twin brothers (patients 2 and 3). No family history was available in two NPS patients and no information could be obtained about the father of patient 5. All patients had renal involvement varying from microscopic hematuria, mild proteinuria, nephrotic syndrome, to end-stage renal disease. Five developed nephrotic syndrome. It was congenital in patient 6 and infantile in patient 4. Various degrees of severity of glomerular lesions were observed, from minimal glomerular changes to nearly diffuse glomerulosclerosis. Typical ultrastructural GBM changes were found in the biopsy specimens from all of the five patients studied by electron microscopy and in the frozen nephrectomy specimen from patient 6. However, in patient 4, no GBM changes were detected at 21 months of age (some fibrillar structures were present in the mesangial matrix) whereas irregular GBM thickening with fibrillar deposits were seen

Table 2. Characteristics of Antibodies Used in the Study

Antibodies	Specificity	Dilution	Source
A: Anti-collagen antibodies			
Rabbit anti- $[\alpha 1(\text{IV})2 \alpha 2(\text{IV})]$	Type IV collagen $[\alpha 1(\text{IV})2 \alpha 2(\text{IV})]$	1/60	Novotec, Lyon, France
Mouse mAb anti- $\alpha 1(\text{IV})$	$\alpha 1(\text{IV})\text{NC1}$	1/2	Wieslab AB, Lund, Sweden
Mouse mAb M3F7	Type IV collagen $[\alpha 1(\text{IV})2 \alpha 2(\text{IV})]$	1/200	Hybridoma Bank, Iowa City, IA
Mouse mAb3	$\alpha 3(\text{IV})\text{NC1}$	1/40	Wieslab AB, Lund, Sweden
Mouse mAb 85	$\alpha 4(\text{IV})\text{NC1}$	1/4	M.M. Kleppel, Minneapolis, MN ³⁴
Rat mAb H43	$\alpha 4(\text{IV})\text{NC1}$	1/25	Y Sado, Okayama, Japan ³⁵
Rat CFT-45325	$\alpha 2-\alpha 5 (\text{IV})\text{NC1}$	1/1	Shigei Medical Research Institute, Okayama, Japan
Mouse mAb A7	$\alpha 5(\text{IV})\text{NC1}$	1/4	Wieslab AB, Lund, Sweden
Goat anti-collagen I	Type I collagen	1/10	Southern Biotechnology, Birmingham, AL
Goat anti-collagen III	Type III collagen	1/5	Southern Biotechnology, Birmingham, AL
Goat anti-collagen V	Type V collagen	1/10	Southern Biotechnology, Birmingham, AL
Mouse mAb 5C6	Type VI collagen	1/1000	Hybridoma Bank, Iowa City, IA
B: Other antibodies			
Rabbit anti-fibronectin	Fibronectin	1/80	Novotec, Lyon, France
Rabbit anti-fibrinogen	Fibrin/fibrinogen	1/100	Dako AS, Glostrup, Denmark
Mouse mAb 1924	Laminin $\alpha 5$	1/100	Chemicon International, Temecula, CA
Mouse mAb C4	Laminin $\beta 2$	1/20	Hybridoma Bank, Iowa City, IA
Mouse mAb 458	Agrin HSPG	1/100	Chemicon International, Temecula, CA
Mouse mAb 5C11	GLEPP1	1/100	BioGenex, San Ramon, CA
Rabbit anti-podocin	Podocin	1/1000	Rosselli et al ³⁶
Rabbit anti-nephrin	Nephrin	1/500	Tryggvason et al ³⁷
Mouse mAb G1D4	Synaptopodin	1/10	Progen Biotechnik GMBH, Heidelberg, Germany
Mouse mAb V9	Vimentin	1/100	Beckman Coulter, Brea, CA
Mouse CD49c	Alpha-3 integrin	1/100	Beckman Coulter, Brea, CA

Cyanine2 or fluorescein isothiocyanate-conjugated AffiniPure goat or donkey anti-rabbit IgG and cyanine3-conjugated AffiniPure donkey anti-mouse IgG antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). Vectastain Elite ABC kit, avidin-biotin blocking kit, and diaminobenzidine were from Vector Laboratories (Burlingame, CA).

5C11 (BioGenex, San Ramon, CA) is a monoclonal antibody reacting with human GLEPP1 protein, a transmembrane protein tyrosine phosphatase found only in the podocytes.

G1D4 (Progen Biotechnik GMBH, Heidelberg, Germany) is a monoclonal antibody recognizing synaptopodin, an actin-associated protein in differentiated podocytes and in a subset of telencephalic synapses.

Vimentin antibody (clone V9) (Beckman Coulter, Villepinte, France) is a monoclonal antibody reacting with the vimentin 57-kd intermediate filament protein without any cross-reaction with other intermediate filament proteins such as cytokeratin, desmin, neurofilaments, or glial fibrillary acidic protein.

Biotinylated horse anti-mouse immunoglobulins, Vectastain Elite ABC kit, diaminobenzidine, and VIP substrates were from Vector Laboratories (Burlingame, CA).

at 5 years. No ultrastructural examination was performed on end-stage kidneys from patient 3. Frozen tissues (four specimens) were used for the immunohistological analysis of podocyte proteins (podocin, nephrin, CD2AP, $\alpha 3$ -integrin) and analysis of the glomerular extracellular matrix. Paraffin-embedded tissues (seven specimens) were used for immunodetection of type III collagen, and analysis of podocyte proteins podocin, synaptopodin, GLEPP1, and vimentin, detectable after fixation and paraffin-embedding. Four normal kidneys (from humans 1 to 51 years of age), unsuitable for transplantation because of vascular problems, were used as controls. In addition, nephrectomy specimens obtained before renal transplantation from 10 patients affected with various types of nephropathies (renal hypoplasia, thrombotic microangiopathy, cystinosis, nephronophthisis) were studied for the comparative analysis of changes in the glomerular extracellular matrix of sclerotic glomeruli. None of these kidneys were from patients with Alport syndrome or with steroid-resistant nephrotic syndrome. Specimens were either immediately snap-frozen in liquid nitrogen using OCT compound (Miles Laboratories Inc., Naperville, IL) and stored at -80°C until use, and/or fixed in 4% formalin or Dubosq-Brazil medium before embedding.

Mutation Screening

Two NPS patient DNA samples were available for *LMX1B* mutation analysis. Genomic DNA was extracted from whole blood or renal tissue in patients 3 and 6, respectively, using standard protocols.³³ Screening of the *LMX1B* gene for mutations was performed by single-strand conformation polymorphism analysis, as previously described.²⁶ When a band shift was detected, the corresponding fragment was sequenced bidirectionally to identify the nature of the mutation. In case no mutations were detected by single-strand conformation polymorphism analysis, all exons were sequenced. Automated DNA sequencing was performed on an Abi Prism 377 (PE Biosystems, Nieuwerkerk a/d Yssel, The Netherlands) using dye terminator chemistry.

Immunohistology

Antibodies (Table 2)

Analysis of the glomerular extracellular matrix was performed using antibodies against types I, III, IV, V, and VI collagen; the $\alpha 1$, 2, 3, 4, and 5 chains of type IV collagen;

fibronectin; laminin $\alpha 5$ and $\beta 2$ chains; agrin; and fibrin. Podocyte proteins were studied using antibodies against GLEPP1, synaptopodin, nephrin, podocin, CD2AP, $\alpha 3$ -integrin, and vimentin.

Cyanine2 or fluorescein isothiocyanate-conjugated AffiniPure goat or donkey anti-rabbit IgG and cyanine3-conjugated AffiniPure donkey anti-mouse IgG antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). The Vectastain Elite ABC kit, avidin-biotin blocking kit, and diaminobenzidine were from Vector Laboratories (Burlingame, CA).

For immunoelectron microscopy, normal human serum adsorbed goat anti-rabbit IgG conjugated with 10-nm colloidal gold particles (GAR-gold 10 nm), staphylococcal protein A conjugated with 15-nm colloidal gold particles (ptA-gold 15 nm) were obtained from BioCell (Cardiff, UK). Rabbit anti-protein A was obtained from Sigma (St. Louis, MO).

Immunofluorescence and Immunoperoxidase Staining

Immunofluorescence labeling was performed on 3- μ m-thick cryostat sections fixed in acetone for 10 minutes and incubated for 20 minutes with 10% normal swine serum in PBST [0.01 mol/L phosphate-buffered saline (PBS) containing 0.05% Tween-20] for blocking nonspecific binding. After incubation for 1 hour at room temperature with primary antibodies diluted in the same buffer, sections were rinsed three times in PBS and incubated for 30 minutes with conjugated anti-rabbit or anti-mouse antibodies diluted to 1 to 40 to 1 to 80 in PBS. A mounting medium (Fluorepre; BioMérieux, Lyon, France) was used to delay fluorescence quenching. Labeling was examined with a Leitz Orthoplan microscope equipped for light, fluorescence, and phase contrast microscopy.

Before incubation with mouse monoclonal antibodies mAb3, mAb85, and mAbA7, and rat H43, the slides were pretreated with 0.1 mol/L of glycine and 6 mol/L of urea, pH 3.5, for 10 minutes, to reveal hidden antigenic determinants.³⁸ They were then rinsed with distilled water and processed as described above.

Immunoperoxidase staining for type III collagen was performed using the Vectastain Elite ABC kit and diaminobenzidine substrate as previously described.³⁹ Three- μ m-thick deparaffinized sections were pretreated by microwave heating in a urea solution (2×5 minutes in 0.8 mol/L of urea, pH 6.4). After washing in fresh buffer (0.01 mol/L of PBS, pH 7.4), endogenous biotin was blocked by the biotin blocking agent, according to the instructions of the manufacturer. Then sections were incubated for 1 hour at room temperature in a moist chamber with the primary antibody in PBST. They were washed in PBS and incubated with biotinylated secondary antibody for 30 minutes. For quenching the endogenous peroxidase, sections were treated with 3% hydrogen peroxide in methanol for 5 minutes and then washed in PBS for 20 minutes. They were then incubated for 30 minutes with Vectastain Elite ABC reagent. After washing, final staining of the sections by diaminobenzidine was monitored under the microscope. Tissue sections directly incubated

with the secondary antibodies or incubated with the pre-immune rabbit sera served as control experiments.

Immunogold Electron Microscopy

Renal biopsy samples from two patients were fixed in 4% buffered paraformaldehyde and embedded in LR-White. The resin was polymerized at -10°C . Ultrathin sections were cut, mounted on nickel grids coated with collodium and carbon films, and processed for immunocytochemistry.

For immunolabeling with polyclonal rabbit anti-collagen I, III, and IV antibodies, tissue sections were first incubated on a drop of 0.01 mol/L of PBS, pH 7.4, containing 0.5% bovine serum albumin (BSA), 0.1% gelatin (G), and 0.05% Tween 20 (T) (PBS-BSA-G-T buffer), then transferred to a drop of primary antibody diluted in the same buffer and incubated at room temperature for 120 minutes. The grids were rinsed with PBS-BSA-G-T buffer and then incubated with goat anti-rabbit IgG (GAR-gold 10 nm). Alternatively, they were incubated with protein A conjugated with 15-nm colloidal gold particles (ptA-gold 15 nm) for 45 minutes at room temperature before incubation with anti-protein A, then reincubation with ptA-gold 15 nm for 45 minutes. Subsequently, they were washed with PBS, postfixed with 2% glutaraldehyde, and dried. After staining with uranyl acetate and lead citrate, the sections were examined with a Siemens Elmiskop 101 electron microscope.

Three normal kidney tissues not used for transplantation were tested as controls for evaluation of the normal distribution of the antigens. Incubation of the sections with normal rabbit or mouse serum instead of the primary antibodies, or direct incubation with secondary antibody or the protein A-gold alone were performed as control experiments, and no positive results were obtained.

Results

Mutations in the LMX1B Gene

Screening of mutations in the *LMX1B* gene was performed by single-strand conformation polymorphism analysis and direct sequencing in two NPS patients. Patient 3, one of the twin brothers, was heterozygous for a T $\rightarrow$ A transition in exon 5 that creates a valine to aspartate missense mutation (V240D) in the homeodomain. Patient 6 was heterozygous for a G $\rightarrow$ A transversion in exon 2, resulting in a cysteine to tyrosine missense mutation (C36Y) in the first LIM domain of the protein that may abolish the Zn(II) binding site.

Immunofluorescence/Immunoperoxidase Labelings

Extracellular Matrix Proteins

Normal Controls: In the normal kidney, antibodies to the [$\alpha 1(\text{IV})2 \alpha 2(\text{IV})$] molecule, the $\alpha 1$ or the $\alpha 2$ chain of type IV collagen stained all extraglomerular basement mem-

Figure 1. Immunohistological analysis of the expression of type IV collagen chains (immunofluorescence) and type III collagen (immunofluorescence and immunoperoxidase) in glomeruli from normal controls and NPS patients. Two stages of glomerular lesions are studied: normal appearing glomeruli (NPS1) and sclerotic glomeruli (NPS2). No decrease in the level of expression of the $\alpha 3$ to $\alpha 5(\text{IV})$ chains is observed in NPS patients. Thin segmental labeling of the GBM with anti-type III collagen antibodies is seen in preserved glomeruli. Massive accumulation of type III collagen is observed in completely sclerotic glomeruli.

branes (BM). Within the glomerular tuft, they strongly labeled the mesangial matrix and gave a thin linear staining of the GBM (Figure 1). Antibodies to the $\alpha 3$ to $\alpha 5$ chains of type IV collagen stained linearly the GBM and the distal tubule BM. In addition the $\alpha 5(\text{IV})$ chain was expressed in the Bowman's capsule and collecting duct BM. Antibodies to type VI collagen and fibronectin stained the mesangial matrix and faintly labeled the sub-endothelial aspect of the GBM. They also stained peritubular capillaries (data not shown). Types I, III, and V collagen were present in the interstitium and absent from glomeruli (Figure 1). Antibodies against $\alpha 5$ and $\beta 2$ laminin chains and agrin heparan sulfate proteoglycan gave a linear staining of the GBM. Bowman's capsules and extraglomerular BM were also stained with antibodies to laminin chain $\alpha 5$ and agrin whereas the $\beta 2$ chain had a restricted GBM and arterial distribution (data not shown).

End-Stage Non-NPS Kidneys: Globally the same normal distribution of extracellular matrix proteins was observed in preserved glomeruli of end-stage renal disease patients not affected with NPS. In sclerotic ones, persistent and strong labeling of the wrinkled GBM was observed with antibodies against the $\alpha 3$, $\alpha 4$, and $\alpha 5$ chains of type IV collagen, the $\alpha 5$ and $\beta 2$ chains of laminin and agrin. Decrease or disappearance of mesangial labeling with anti-type IV collagen [$\alpha 1(\text{IV})2$ $\alpha 2(\text{IV})$] antibodies contrasted with the strong labeling with antibodies to type VI collagen. Focal and segmental labeling of sclerotic glomeruli could be observed with antibodies to types I, III, and V collagen (data not shown).

NPS Kidneys: The intensity and distribution of glomerular labeling with anti-type IV collagen chains ($\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, and $\alpha 5$) was similar to that observed in controls in nonsclerotic and in sclerotic glomeruli (Figure 1). In particular the anti- $\alpha 3$, - $\alpha 4$, and - $\alpha 5$ (IV) antibodies gave a strong linear staining of the GBM and a moderate labeling of distal tubule basement membranes. Labeling of collecting duct BMs with the anti- $\alpha 5(\text{IV})$ antibodies was also normal when compared to controls. In sclerotic glomeruli, labeling with antibodies to the $\alpha 3$, $\alpha 4$, and $\alpha 5$ chains of type IV collagen was especially strong, similar to the one seen in control sclerotic kidneys. In contrast poor labeling of the tuft was observed with the anti- $\alpha 1$ and anti- $\alpha 2$ antibodies. The distribution of noncollagenous components of the GBM ($\alpha 5$ and $\beta 2$ laminin, and agrin) was also preserved (data not shown). No significant differences in the glomerular distribution of types I, V, or VI collagen were observed in sclerotic glomeruli compared to sclerotic controls (data not shown).

In contrast, by immunofluorescence and immunoperoxidase, an irregular and discontinuous labeling of the GBM with anti-type III collagen antibodies was seen in normal appearing glomeruli (Figure 1). This labeling varied in extension and intensity among glomeruli, and within a glomerulus, from one capillary wall to another. In sclerotic glomeruli large amounts of type III collagen were focally seen within segments of the tuft (Figure 1). Within the interstitium, the intensity and extension of type

III collagen labeling paralleled the severity of interstitial fibrosis.

Fibrin/Fibrinogen

In NPS kidneys, as in controls, no specific labeling of the GBM or of the mesangial matrix was observed with anti-fibrin/fibrinogen antibodies (data not shown).

Podocyte Proteins

Normal Controls

In normal mature kidneys, specific podocyte labeling was seen with antibodies to nephrin, podocin, CD2AP, GLEPP-1, and synaptopodin. Labeling was observed along the GBM (Figures 2 and 3). Other renal structures were negative, except for CD2AP that was expressed in tubular cells.

Strong labeling of podocyte cytoplasm was observed with anti-vimentin antibodies (Figure 3). It was also seen in other glomerular cells as well as in interstitial and vascular, muscular, and endothelial cells. Tubular cells were vimentin-negative.

Antibodies against the $\alpha 3$ chain of the integrin $\alpha 3\beta 1$ gave a strong labeling of the podocytes along the GBM. It was also expressed, at a lower level, by distal tubular epithelial cells (Figure 3).

End-Stage Non-NPS Kidneys

Normal labeling of preserved podocytes along sclerotic or nonsclerotic glomeruli was observed with all antibodies against podocyte proteins.

NPS Kidneys

Normal podocyte labeling was observed for all antibodies tested (Figures 2 and 3). Especially no qualitative or quantitative difference in podocin, CD2AP, or nephrin labeling was detected between controls and nail-patella kidneys (Figure 2). In end-stage kidneys with severe glomerular changes, preserved podocytes were clearly labeled with all antibodies.

Electron Microscopy

The six renal biopsies from five patients as well as the nephrectomy specimen from patient 6 were studied by electron microscopy. In patients 5, 6, and 7, many glomeruli were completely or partially sclerotic. In less damaged glomeruli of these patients as well as in glomeruli of patients 1 and 2, which looked normal by light microscopy, typical ultrastructural abnormalities of the GBM were observed. They were always focal and their extent varied within and between glomeruli. They consisted in irregular thickening of the GBM-containing electron lucent zones (Figure 4) or dense fibrillar material distributed in the lamina densa but also observed in subepithelial or subendothelial location. Fibrillar bundles and their

Figure 2. Immunohistological analysis of noncollagenous podocyte gene expression in glomeruli from normal controls and NPS patients. Two stages of glomerular lesions are studied: normal appearing glomeruli (NPS1) and partially sclerotic glomeruli (NPS2). No difference in the level of expression of nephrin, podocin, or CD2AP is observed between control and NPS1 glomeruli. In NPS2 glomeruli, expression of nephrin, podocin, and CD2AP is seen in preserved podocytes.

periodicity were better seen after phosphotungstic impregnation (Figure 4). Clusters of fibrils were also present in the mesangial areas. Along patent capillary lumens, podocytes were usually normal or showed focal efface-

ment of foot processes; slit diaphragms were otherwise preserved. Occasionally, the rough endoplasmic reticulum was increased and dilated. Most endothelial cells were normal, some of them were slightly swollen with focal loss of fenestration

In patient 4, no GBM changes were detected on the first renal biopsy. Only a few fibrillar structures were seen in the slightly increased mesangial matrix. Focal effacement of foot processes was present but most pedicels and slit diaphragms were preserved. Severe and diffuse glomerular lesions were present on the second biopsy specimen. They consisted in diffuse mesangial hypercellularity and matrix increase associated with marked thickening of the capillary wall because of focal double-contours and accumulation of basement membrane-like material in subendothelial location. Abundant aggregates of fibrils were seen in the GBM and the mesangial matrix. Podocytes were enlarged, frequently vacuolized, with focal effacement of foot processes. They were rich in rough endoplasmic reticulum. Glomerular and arteriolar endothelial cells were swollen.

Figure 3. Immunohistological analysis of podocyte gene expression in glomeruli from normal controls and NPS patients. The level of expression of synaptopodin, Glepp 1, $\alpha 3$ integrin, and vimentin is similar in controls and NPS patients.

Electron Microscopy: Immunogold Labeling

Type I, III, V, and VI Collagen

In normal control kidneys, using anti-type I, III, or V collagen antibodies, gold particles were specifically distributed on interstitial bundles of fibrillar collagen. No

Figure 4. Ultrastructural changes in NPS patients. **A:** The GBM is markedly thickened and has a moth-eaten appearance (**arrows**) (lead citrate and uranyl acetate staining). **B:** The fibrillar material is clearly seen after phosphotungstic acid impregnation. **Inset:** On higher magnification the fibrils have the characteristic striated periodicity of interstitial collagen. Interestingly the lesions observed in this patient had a focal distribution: several GBM segments were strictly normal. Original magnifications: $\times 17,000$ (**A**); $\times 15,000$ (**B**); $\times 27,500$ (**inset**).

specific labeling was detected in glomeruli. Using anti-type VI collagen antibodies, gold particles were seen in the glomeruli, on the mesangial matrix, and faintly along the subendothelial space of the GBM.

The same distribution was observed in NPS kidneys for types I, V, and VI antibodies. However, with anti-type III antibodies, in addition to interstitial labeling, gold particles were observed focally within the GBM and the mesangial matrix (Figure 5). They were clearly localized on the fibers or within the clear vacuoles distributed within the thickness of the GBM or the mesangial matrix (Figure 5; A to C) (after parafor-

maldehyde fixation without postfixation with osmium tetroxide, the NPS GBM often have a moth-eaten appearance, the fibrillar inclusions being frequently unstained). No specific labeling of the GBM fibers was obtained with the other anti-collagen antibodies.

Discussion

Ultrastructural abnormalities of the GBM are consistently found in NPS patients, with or without renal symptoms.⁸⁻¹² The NPS GBM was initially described as thick-

Figure 5. Type III collagen immunogold labeling in NPS patients. **A:** Within the GBM, the dense material as well as the clear vacuoles are specifically labeled with the antibody. No gold particles are seen in the adjacent basement membrane. **B:** Numerous gold particles are seen on the mesangial matrix. The paramesangial basement membrane is not labeled. **C:** Higher magnification underlines the close association between the gold particles and the fibrillar material. Original magnifications: $\times 9000$ (**A**); $\times 12,000$ (**B**); $\times 27,000$ (**C**).

ened and moth eaten by Del Pozo and colleagues.⁸ It was subsequently shown that the electron-lucent areas contained a dark fibrillar material, inconsistently stained with uranyl acetate and lead citrate, but always visible after phosphotungstic acid impregnation. In Alport syndrome glomerulopathy, a hereditary type IV collagen disease, characterized by thickening and fraying of the GBM, such fibrillar structures are never observed. Fibrils show the periodicity of interstitial collagen,⁹ however, their precise nature had not been determined. It has been suggested that fibrinogen deposition could be the first step of the lesion because of its finding in glomeruli of a spontaneously aborted fetus from a NPS mother with

platelet defect.¹³ In our study, as in most reports,^{10,40,41} we did not find any fibrin deposit in the GBM. We used antibodies to types I, III, V, and VI collagen to examine the deposits at the light and electron microscopic levels. By light microscopy, we showed the presence of type III collagen within the GBM, with an irregular focal and segmental distribution. By electron microscopy, the labeling was seen on the fibrils located in the electron lucent vacuoles present in the GBM and the mesangial matrix. No labeling of these structures was observed with antibodies recognizing other fibrillar collagens. These data indicate that the ultrastructural hallmark of NPS is because of the abnormal distribution of type III collagen within the GBM.

The presence of type III collagen within the GBM of NPS patients may be because of the regulation of type III collagen gene by *LMX1B*. Alternatively, it could be secondary to structural GBM abnormalities. However the finding of type III collagen in the GBM of asymptomatic patients whose glomeruli are normal by light microscopy strongly suggests that it is a primary lesion.¹⁰ For this reason, we believe that a down-regulation of *COL3A1* by *LMX1B*, either directly or indirectly, is likely. Further experiments are required to test this hypothesis. The pathogenic role of the aberrant GBM expression of type III collagen known to accumulate in nonglomerular fibrotic processes, remains to be determined because the GBM lesions have been observed in NPS patients with or without any renal symptom.^{10,11,41} Conversely, it was not detected in one of our patients biopsied at 21 months of age because of severe nephrotic syndrome and was not described in mouse models of NPS that develop a severe glomerular disease and die at day 1 with renal abnormalities and diffuse effacement of foot processes.^{21,22}

Absence of GBM binding of a monoclonal antibody directed toward the Goodpasture antigen [the noncollagenous domain of $\alpha 3(\text{IV})$], was described in two NPS patients, 40 and 22 years old, respectively, with mild proteinuria and normal renal function.⁴² The labeling was performed on formalin-fixed, paraffin-embedded renal biopsies and the expression of the other type IV collagen chains was not studied. Likewise, the GBM expression of the $\alpha 4$ and $\alpha 3$ chains of type IV collagen is strongly reduced in *Lmx1b*-null mice and *LMX1B* was shown to bind *in vitro* regulatory elements in *COL4A4*.²⁰ These observations suggest that, similarly to the findings in recessive Alport syndrome because of *COL4A3* or *COL4A4* mutations, dysregulation in the podocyte synthesis of $\alpha 3(\text{IV})$ and $\alpha 4(\text{IV})$ collagen chains contributes to GBM changes and renal pathology in NPS patients. However, at variance with murine, canine, or human autosomal recessive Alport syndrome,^{38,43–45} the $\alpha 5(\text{IV})$ chain expression is preserved in the GBM of *Lmx1b*^{−/−} mice. In the normal GBM, the three chains are strongly co-expressed and form a distinct network characterized by loops and super coiled helices that are stabilized by disulfide bonds.^{46,47} The absence of one of these chains in Alport syndrome, when the corresponding gene is mutated, prevents the assembly of the two other chains and the organization of the network, and results in the absence of all three $\alpha 3$, $\alpha 4$, and $\alpha 5(\text{IV})$ chains. In our

study, performed on frozen kidney tissues from NPS patients, heterozygous for *LMX1B* mutation, and affected with a severe glomerular disease, the $\alpha 3$, $\alpha 4$, and $\alpha 5(IV)$ chains were strongly expressed in the GBM, without any visible difference with the expression in controls. In another NPS patient, the occurrence of Goodpasture syndrome was an indirect demonstration of the presence of the $\alpha 3(IV)$ chain in the GBM.⁴⁸ In heterozygous *Lmx1b*+/- mice, a decreased expression of renal *Col4a3* and *Col4a4* mRNAs has been shown by semi-quantitative reverse transcriptase-polymerase chain reaction²⁰ but these mice did not develop any renal symptom. Moreover, patients with heterozygous mutations of *COL4A3* or *COL4A4*, and reduced synthesis but normal distribution of the $\alpha 3$, $\alpha 4$, and $\alpha 5(IV)$ chains frequently show thin GBM.⁴⁹ Thin GBM was not observed in our series of NPS patients and never reported in the literature, perhaps because of the persistence of more than 50% expression of both chains, as observed in *Lmx1b*+/- mice.²⁰ In addition, contrary to the findings in mice, the expression of podocin and CD2AP, two podocyte proteins important for podocyte function as demonstrated by the occurrence of early and severe nephrotic syndrome when they are mutated,^{31,32} was found to be normal in NPS patients. Again, a slight reduction of these proteins may escape detection by immunohistochemical methods, although contributing to progressive renal dysfunction. Such a level of expression may be sufficient for proper renal development in humans, whereas kidneys in *Lmx1b*-/- mice show severe developmental defects: they have small kidneys with smaller than normal glomeruli. The glomerular endothelial cells are poorly fenestrated and the podocytes have an immature appearance: they are cuboidal, lack foot processes and slit diaphragms and are connected by structures resembling adherens junctions.^{21,22} In NPS patients, in our experience as in other reported data, the glomerular endothelial cells were normal and podocytes were large fully differentiated cells with long cytoplasmic expansions running toward the capillaries and dividing into foot processes. Slit diaphragms were clearly identified. Effacement of foot processes was observed only focally along severely affected GBM segments, or in patients with nephrotic syndrome in association with massive proteinuria.

Globally, in NPS patients heterozygote for *LMX1B* mutation, we show that the fibrillar material observed in the thick GBM segments is type III collagen. We did not detect any of the GBM or podocyte abnormalities described in *Lmx1b*-/- mice: no specific podocyte changes were observed by electron microscopy, by immunofluorescence, CD2AP and podocin appeared to be normally expressed by the podocytes and the distribution of type IV collagen chains $\alpha 3$ and $\alpha 4$ was similar to that found in normal kidneys. However, a moderate reduction of expression of several genes playing a role in the maintenance of the structure and function of the glomerular filtration barrier, and escaping detection by immunohistochemical methods, may explain the glomerular disease in the humans. But, at variance with heterozygous *Lmx1b*+/- mice that did not develop any symptom, our seven patients with obvious nail and bone abnormalities,

had a glomerular disease that progressed to end-stage renal disease in five. It may be hypothesized that the presence of type III collagen in the glomerular extracellular matrix is an additional pathogenic factor involved in kidney disease progression.

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