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Nephrin expression is increased in anti-Thy1.1-induced glomerulonephritis in rats☆

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Abstract

Nephrin is an important constituent of the glomerular filtration barrier and alteration of its expression is associated with severe proteinuria. In this study we show that injection of an anti-Thy1.1 antibody in rats not only induces a mesangioproliferative glomerulonephritis associated with increased proteinuria, but also leads to a sustained increase of nephrin mRNA and protein expression in renal glomeruli over a time period of 29 days. In contrast, podocin and CD2AP, two proteins shown to interact with nephrin in the slit diaphragm, are acutely downregulated at days 3–7 and, thereafter, recovered again to normal levels after 29 days. Interestingly, immunofluorescence staining of kidney sections at day 10 of the disease shows a highly heterogeneous pattern, in that some podocytes show complete absence of nephrin, whereas others show highly accumulated staining for nephrin compared to control sections, which in total results in an increased level of nephrin per glomerulus. In summary, our data show that in the course of mesangioproliferative glomerulonephritis in rats, an upregulation of nephrin expression occurs with a concomitant transient downregulation of podocin and CD2AP which may account for a highly dysregulated filtration barrier and increased proteinuria.

Keywords: Nephrin; Podocin; CD2AP; Inflammatory disease; Thy1.1; Glomerulonephritis; Podocytes; Kidney

Glomerular visceral epithelial cells, also denoted as podocytes, elaborate regularly spaced, interdigitated foot processes, and form the filtration slit. The foot processes are connected by a thin membrane-like slit diaphragm which plays an important role in the regulation of the glomerular filtration barrier [1,2]. Upon podocyte damage which can be triggered by various factors including oxidative and inflammatory stress [3,4] foot processes undergo a typical flattening which results in a destruction of the filtration barrier and proteinuria which is a hallmark of many kidney diseases [1,2,5–7].

It has become clear that the recently identified podocyte-specific protein nephrin plays an important structural role in the normal kidney filtration unit [8–10]. Deficiency of nephrin due to mutations in the *NPHS 1* gene, as it is found in the congenital nephrotic syndrome of the finnish type (CNSF), results in massive proteinuria and renal failure [8–10]. Biochemically, nephrin was identified as a 185-kDa glycosylated transmembrane protein possessing eight immunoglobulin-like domains and one fibronectin-like domain in the

☆ *Abbreviations:* CD2AP, CD2-associated protein; CNSF, congenital nephrotic syndrome of the finnish type; ECL, enhanced chemiluminescence; FSGS, focal segmental glomerulosclerosis; GAPDH, glyceraldehydes 3-phosphate dehydrogenase; GN, glomerulonephritis; IL-1β, interleukin-1β; PAS, periodic acid–Schiff reaction; PBS, phosphate-buffered saline solution; TBS, Tris-buffered saline solution; THY1-GN, anti-Thy1-induced glomerulonephritis; TNFα, tumor necrosis factor-α.

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extracellular part and a short intracellular C-terminus consisting of 164 amino acids. Interestingly, within this intracellular C-terminal end, several putative phosphorylation sites are found, suggesting that nephrin is able to trigger activation of intracellular signal transduction cascades. Very recently, evidence was presented that nephrin undergoes homophilic interaction which may represent the long-searched initiating stimulus for nephrin-triggered signal transduction [11].

Due to the very early finding that a mutation or deficiency of nephrin correlates with proteinuria, it has become a more or less dogmatic issue that a reduction of nephrin expression leads to disturbed ultrafiltration. Nevertheless, we have previously shown that in a cell culture model of human embryonic kidney cells (A293) but also in primary human podocytes, proinflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα) lead to an upregulation of nephrin expression on mRNA and protein level [12]. This led us to speculate that proteinuria not necessarily correlates with decreased nephrin expression but that under certain conditions also an increased nephrin expression may cause a dysregulation of the filtration complex and subsequent proteinuria. To evaluate this observation in vivo, we investigated the change of nephrin, podocin, and CD2-associated protein (CD2AP) expression in the course of a rat model of mesangioproliferative glomerulonephritis (GN). We found a sustained increase in nephrin expression in the course of the disease and this was associated with an increased proteinuria. In contrast, podocin and CD2AP two other proteins in the filtration unit were transiently downregulated and thereafter recovered again.

Materials and methods

Chemicals. Mouse anti-rat Thy-1.1 IgG, clone OX-7 was purchased by BioTrend, Köln, Germany; an affinity purified rabbit anti-rat nephrin antibody was generated as previously described [12] and used for Western blot analysis; a guinea pig anti-mouse nephrin (cross-reacting with rat nephrin) was obtained from Progen Biotechnik GmbH, Heidelberg, Germany, and used for immunocytochemical staining; a rabbit anti-rat podocin antibody was generated by immunizing a rabbit with two synthetic peptides (CEY EES QWT GER DTQ S and QPS GEP EDQ LPT EPPC) based on the sequence of the human podocin (Accession No. NM_004646) coupled to keyhole limpet hemocyanin. The antiserum was affinity purified by using a peptide-coupled Sepharose column. Rabbit anti-CD2AP, rabbit anti-GAPDH, and anti-β-tubulin antibodies were from Santa Cruz, Heidelberg, Germany. Cy3-conjugated affinity purified goat anti-guinea pig antibody was from Chemicon, Hofheim, Germany. TRI-zol was from Invitrogen, Groningen, The Netherlands. Anti-rabbit horseradish peroxidase-linked IgGs, hyperfilms, nitrocellulose membranes, and the enhanced chemiluminescence (ECL) reagents were obtained from Amersham–Pharmacia Biotech Europe GmbH, Freiburg, Germany. The BCA Protein Assay Reagent was obtained from Pierce, Rockford, IL, USA. Standard laboratory rat chow was from Altromin, Lippe, Germany.

Rat model of glomerulonephritis. All animal experimentation was conducted in accordance with the German Animal Protection Act and was approved by the Ethics Review Committee for laboratory animals of the District Government of Muenster, Germany. The anti-Thy1.1-glomerulonephritis (THY1-GN) was induced in adult male Wistar rats weighing 150–180 g (Charles-River, Sulzfeld, Germany) by a single intravenous injection of mouse anti-rat Thy-1.1 IgG, clone OX-7, dissolved in 18 mM sodium phosphate, pH 7.4/0.15 M NaCl (PBS), at a dose of 1 mg/kg body weight. Control animals received a single intravenous injection of PBS only. Kidneys were harvested at 3, 7, 10, 15, 22, and 29 days ($n = 4$ at days 3 and 7 and $n = 5$ for controls and day 10, $n = 3$ animals for other time points) after induction of Thy 1-nephritis. Urine was collected in metabolic cages for 24 h prior to harvesting of the kidneys under free access to water and standard laboratory rat chow. Animals were anesthesized with hexobarbital (150 mg/kg), the kidneys were removed, and glomeruli were isolated RNase-free by differential sieving according to the method of Spiro [13]. Glomeruli from each kidney preparation were washed three times, examined by light microscopy (purity of isolates >95%), counted thrice in a Fuchs–Rosenthal chamber, and processed for Western blotting or RNA isolation. Protein concentration in the urine was determined using the BCA Protein Assay.

Reverse transcriptase-PCR. Total RNA was isolated using guanidinium isothiocyanate solution. Ten micrograms of total RNA was used for reversed transcription (using a First Strand cDNA Synthesis Kit, MBI Fermentas) in 20 μl of volume. The following sequences were performed for PCR (*Taq* DNA Polymerase, recombinant, MBI): 94 °C for 45 s, 58 °C for 1 min, 72 °C for 45 s (with 35 cycles), and final extension at 72 °C for 10 min. Sequences of the primers for analysis of mRNA: rat nephrin: forward: GTT CAG CTG GGA GAG ACT GG; reverse: TTG GAC ATC CAG AGG GAC C; GAPDH: forward: AAT GCA TCC TGC ACC ACC AA; reverse: GTC ATT GAG AGC AAT GCC AGC. PCR products (length: 340 bp for rat nephrin and 470 bp for GAPDH) were run on a 1.0% agarose gel containing 0.5 μg/ml ethidium bromide. The identities of PCR products were confirmed by sequencing on a Genetic Analyzer 310 (Perkin–Elmer).

Northern blot analysis. Total RNA was extracted from isolated glomeruli using TRI-zol. A cDNA probe for rat nephrin was generated by RT-PCR from total rat kidney RNA using random hexanucleotide cDNA primers. The sequences for the primers of the rat nephrin cDNA (Accession No. AF161715) were: forward: TGA AAC CGT GAG CTC CTT CT; reverse: CTG GAT GTT GGT GTG GTC AG, yielding a cDNA product of 910 bp. The cDNA probe for GAPDH was from ATCC (Rockville, MD). Northern blots were performed and analyzed as described previously [14]. For quantification, the Phosphor screens were analyzed by a STORM860 Phosphor Imager using IQ Solutions Image Quant software (both from Molecular Dynamics, Uppsala, Sweden). Each individual mRNA band was normalized for GAPDH to correct for the difference in RNA loading and/or transfer. Values are given as means ± SEM from three Northern blots.

Western blot analysis. Glomerular homogenates from each individual rat kidney containing equal amounts of glomeruli were heated for 5 min at 95 °C in Laemmli buffer, separated on SDS–PAGE (7.5% acrylamide gel), and transferred to nitrocellulose membranes. The membranes were blocked with 3% casein, 1% goat serum, and 0.002% Tween 20 in 10 mM Tris/HCl, pH 7.4/0.15 M NaCl (TBS) and incubated for 90 min at 37 °C with the primary antibodies (rabbit anti-rat nephrin, anti-podocin, anti-CD2AP, anti-GAPDH, and anti-tubulin; each in a dilution of 1:500 with TBS including 1% bovine serum albumin) followed by washing and incubation with second antibody, horseradish peroxidase-coupled goat anti-rabbit IgG (dilution 1:5000), for 90 min at ambient temperature. The samples were visualized by using the ECL Western blotting reagent kit and analysis was performed with IQ Solutions Image Quant software. Values are given as means ± SEM from three Western blots representing the content of

nephrin, podocin or CD2AP in 10^3 of glomeruli or they were normalized to GAPDH or β-tubulin content in the glomerular extracts.

Morphological and immunohistochemical studies. Paraffin-embedded kidney sections (4 μm) were stained with periodic acid–Schiff reaction (PAS). For immunohistochemical studies frozen renal sections were incubated with guinea pig anti-mouse nephrin (1:50) for 1 h at 37 °C. Unspecific staining was blocked with PBS/1% bovine serum albumin/20% goat serum. For visualization Cy3-conjugated affinity purified goat anti-guinea pig antibody was used at a dilution of 1:500. Laser scan microscopy was carried out with a Nikon confocal microscope PCM 2000 (Nikon, Duesseldorf, Germany). The specificity of immunostaining was tested by omitting the primary antibody or by using non-immune serum.

Statistical analysis. Results are expressed as means ± SEM. Statistical analysis was performed by the unpaired Student's *t* test. Significance was accepted at the 5% level.

Results

Previously, we have shown that the proinflammatory cytokines IL-1β and TNF-α are able to induce nephrin mRNA and protein expression in primary cultures of human podocytes and in the human embryonic kidney cell line A293 [12]. We therefore investigated here whether such an upregulation of nephrin is also seen in an in vivo model of renal inflammation. The anti-Thy1.1 mesangioproliferative glomerulonephritis model in rats was chosen [15] and initiated by injection of a single dose of 1 mg/kg body weight anti-Thy1 antibody into rats which leads to a rapid increase of urinary protein loss. A significant enhancement is seen already at day 3 which constantly increased up to day 10 and persisted up to day 29, the latest time point measured (Fig. 1A). By light microscopy of kidney sections of control rats and diseased rats, we clearly observe at day 3, mesangiolytic lesions including microaneurysms. The early mesangiolysis subsequently leads to mesangial hyperproliferation resulting in pronounced glomerular hypercellularity at day 10, while at day 29 glomeruli were in the resolution phase (Fig. 1B).

Whole kidney RNA extracts were subjected to a semiquantitative reverse transcriptase-PCR using specific primers for rat nephrin. As seen in Fig. 2, nephrin mRNA expression starts to increase significantly already after 1 day of anti-Thy1.1 injection and constantly increases over the next 12 days.

To address the change in nephrin mRNA levels in a more specific and quantitative manner Northern blot analysis was performed from isolated glomeruli following the disease course. Again, nephrin mRNA steadily increases and reaches maximal levels between day 10 and day 22, and thereafter slightly declines again (Fig. 3A, upper panel and Fig. 3B). Since for all the experiments on isolated glomeruli the same amounts of glomeruli (10^3) were taken, we detected as an additional loading control GAPDH mRNA expression, which, however, does not change significantly throughout the experiment (Fig. 3A, lower panel).

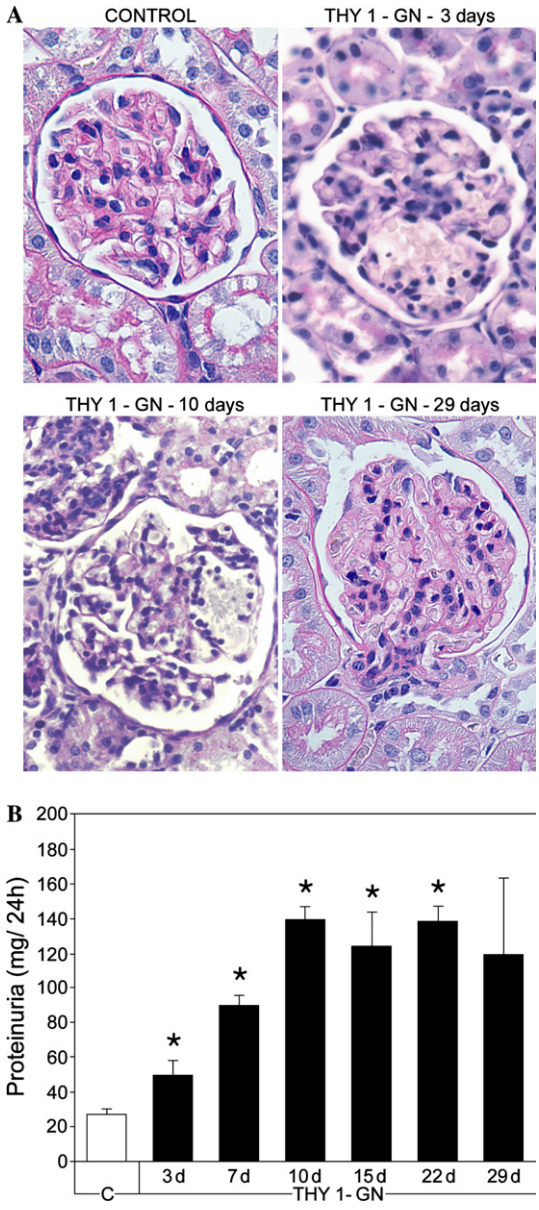

Fig. 1. Morphological changes and proteinuria in the course of anti-Thy1.1-induced glomerulonephritis in rats. (A) Light microscopy of renal cortical sections stained with periodic acid–Schiff stain showing a glomerulus from a healthy control rat vs. a series of glomeruli obtained at days 3, 10, and 29 after induction of THY1-GN, respectively. At day 3, some mesangiolytic lesions including microaneurysms were present and the early mesangiolysis subsequently led to mesangial hyperproliferation resulting in pronounced glomerular hypercellularity at day 10, while at day 29 glomeruli were in resolution phase. Magnification, 400×. (B) Development of proteinuria over the course of THY1-GN. Urine was collected in metabolic cages for 24 h before the kidneys were removed. Values are given as means ± SEM for at least three animals for each time point. *$p < 0.05$ for THY1-GN (black bars) vs. controls (open bar).

Furthermore, Western blot analysis of protein extracts from isolated glomeruli reveals that also nephrin protein expression is markedly upregulated up to day 29 after anti-Thy1.1 injection (Fig. 4A).

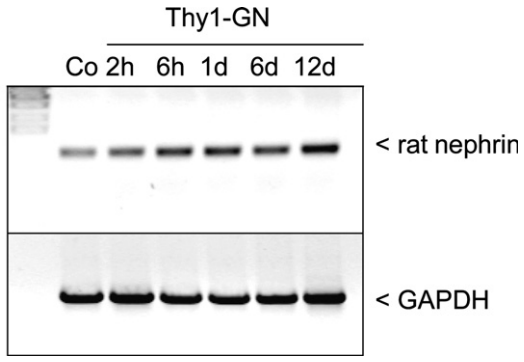

Fig. 2. Nephrin mRNA expression in whole kidney extracts of anti-Thy1.1-treated rats. RNA extracts of total kidneys of control rats (Co) or THY1-GN rats after 2 h up to 12 days were used for semiquantitative reverse transcriptase-PCR using primers for rat nephrin and GAPDH as described in Materials and methods. Samples were separated on a 1.0% agarose gel. Nephrin and GAPDH run at a size of 340 and 470 bp, respectively. Data are representative of three independent experiments.

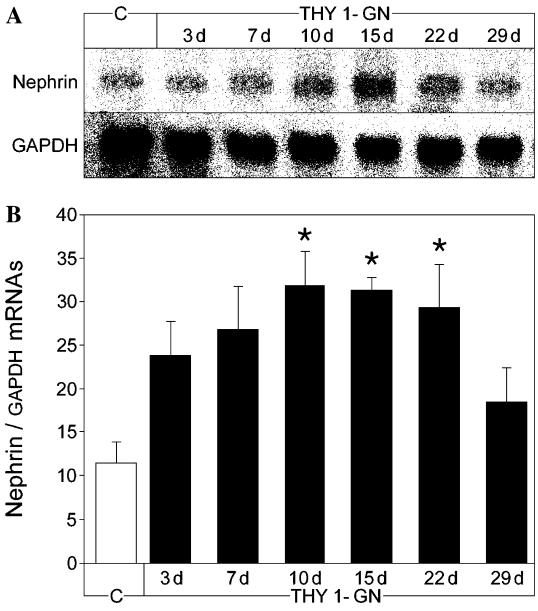

Fig. 3. Expression of nephrin mRNA and protein in the course of anti-Thy1.1-induced glomerulonephritis in isolated glomeruli. Total RNA from glomeruli isolated from individual kidneys (30 μg each) of control rats (C) or THY1-GN rats after the indicated days (3–29 days) was subjected to Northern blot analysis using probes specific for rat nephrin and GAPDH. (A) Data show a representative blot of nephrin (upper panel) and GAPDH (lower panel) out of three independent experiments. (B) Data show the evaluation of nephrin/GAPDH ratios in glomeruli from three individual animals at each time point. Data are given as means ± SEM. $*p < 0.05$ for THY1-GN (black bars) vs. controls (open bar).

To see whether this upregulation of nephrin in THY1-GN is unique for the components forming the filtration unit, we investigated two other proteins of the slit diaphragm, podocin and CD2AP, which both have recently been shown to both interact with nephrin

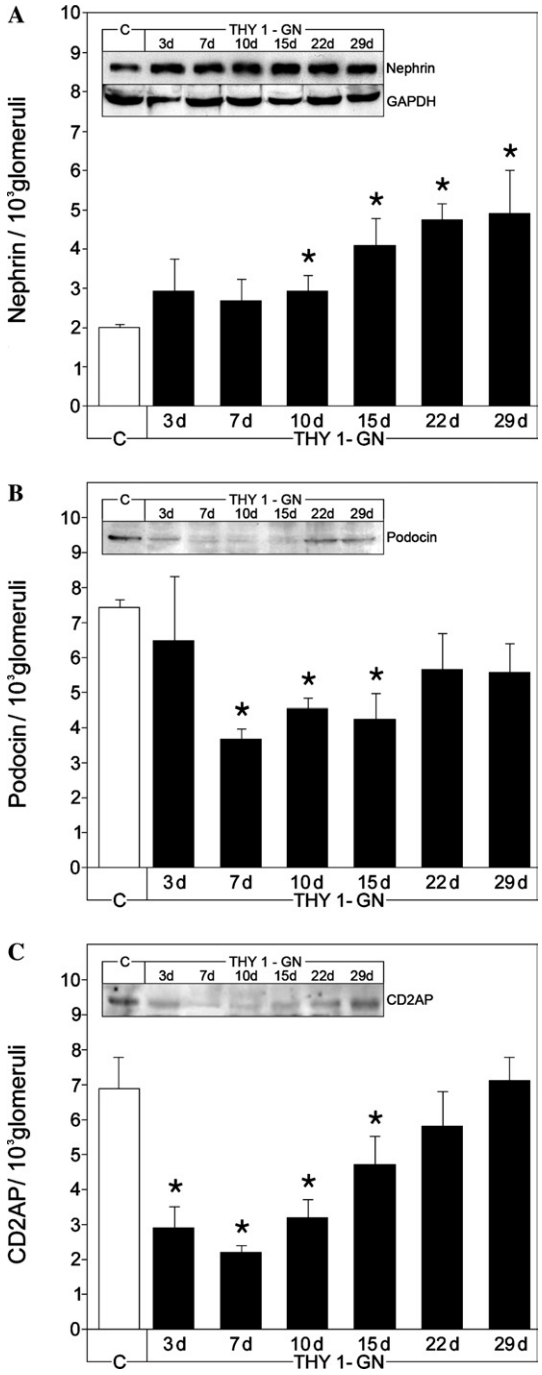

Fig. 4. Protein expression of nephrin, podocin, and CD2AP in the course of anti-Thy1.1-induced glomerulonephritis in isolated glomeruli. Protein lysates of 10^3 glomeruli isolated from individual kidneys of control rats (C) or THY1-GN rats after the indicated days (3–29 days) were separated by SDS–PAGE, transferred to nitrocellulose membrane, and subjected to Western blot analysis using specific antibodies against nephrin (A), podocin (B), CD2AP (C), and GAPDH. Data show the densitometric evaluation of nephrin (A), podocin (B), and CD2AP (C) calculated per 10^3 of glomeruli. Insets in (A,B,C) show one representative Western blot out of at least three independent experiments. The lower panel of inset (A) shows the expression of GAPDH. Data are given as means of the optical density ± SEM from three individual animals at each time point. $*p < 0.05$ for THY1-GN (black bars) vs. controls (open bar).

[16–18]. Interestingly, podocin protein expression is downregulated in the early phase of the disease with a maximal reduction at day 7 and thereafter recover again although after 29 days still a reduced podocin expression exists compared to control healthy glomeruli (Fig. 4B). A similar pattern, i.e., an early downregualtion followed by a normalization, is observed for CD2AP protein expression (Fig. 4C). In contrast, GAPDH is not changed significantly during the course of the disease (see Fig. 4A, inset, lower panel).

In a further step, we investigated the expression of nephrin in kidney sections of diseased rats at day 10 and compared it to control rats by immunofluorescence staining. Surprisingly, we obtained a rather heterogeneous staining pattern. As seen in Fig. 5, red fluorescence reflects podocytes stained with a nephrin antibody and a second Cy3-conjugated anti-guinea pig antibody. Clearly, many podocytes in the diseased sections (Figs. 5B and C) show an accumulated red fluorescence compared to the podocytes in the control healthy kidney section (Fig. 5A). However, some podocytes are completely deficient in nephrin expression (Fig. 5C).

Discussion

During the last few years the view that proteinuria is associated with a decrease of nephrin expression and function in glomerular podocytes has become more or less a dogma. This was mainly based on the finding that a genetically defined deficiency of the nephrin gene as found in the congenital nephrotic syndrome of the finnish type (CNSF) leads to a life threatening proteinuria shortly after birth which can only be cured by kidney transplantation. Also several other forms of proteinuric diseases in experimental animal models as well as in humans have been reported to display reduced nephrin expression although the great variability of the data does not provide a conclusive view.

Thus, puromycin aminonucleoside nephrosis in rats is associated with reduced nephrin and podocin expression [19], but with increased soluble nephrin in the urine [20]. A similar reduction of glomerular nephrin expression is observed in the model of passive Heymann nephritis (PHN) in rats [21]. However, mice injected with an anti-nephrin antibody, which triggers massive proteinuria, have only a modestly reduced nephrin protein expression in podocytes [22] and anti-Thy1 antibody-injected mice, which develop a mesangioproliferative GN show no change in slit pore proteins [23]. Moreover, streptozotocin-induced diabetic rats even show an increased nephrin staining during several weeks of follow-up [24].

In humans the reported data are even more controversial. In this respect, Furness et al. [25] and Doublier et al. [26] showed that nephrin mRNA expression is significantly lowered in focal segmental glomerulosclerosis (FSGS), minimal change GN, and membranous GN, whereas no change was seen in IgA-GN. Contrary to this, others reported no change in the levels of nephrin in FSGS and minimal change GN, but reduced extracellular nephrin expression in IgA nephropathy [27,28]. However, when measuring intracellular nephrin expression in IgA-GN no change was found [27]. Rather, it seems as if intracellular nephrin expression increases in immunocytochemical stainings during IgA-GN and only podocin expression convincingly decreases [29]. More consistent data were obtained in diabetic nephropathy (DN) patients. By investigating differentially regulated genes in kidneys from DN patients compared to healthy kidneys, nephrin was identified as one of the downregulated genes [30]. However, when classifying DN into mild forms and severe forms, only patients with the severe form of DN had significantly reduced nephrin mRNA expression [31].

Although most of these studies have solely investigated nephrin expression on the mRNA level, it should be noted that the other structural components of the slit diaphragm may be equally important as nephrin for normal ultrafiltration. Thus, not only nephrin-deficient mice develop dramatic proteinuria [10,32], but the depletion of podocin leads to a similar massive loss of protein [33].

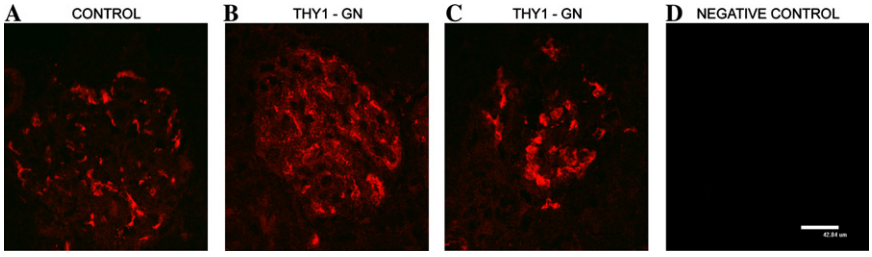

Fig. 5. Immunofluorescent staining of nephrin in kidney sections of anti-Thy1.1-induced GN in rats. Kidney sections of control rats (A) or THY1-GN at day 10 (B–D) were subjected to immunofluorescence staining of nephrin (A–C) as described in Materials and methods. (D) Represents a negative control for the immunostaining of nephrin by omitting the primary antibody. The staining pattern for nephrin in THY1-GN varied from glomerulus to glomerulus revealing either overexpression of nephrin in all podocytes (B) or enhanced staining in some podocytes, while some parts of podocytes were negative for nephrin (C).

Moreover, also CD2AP (−/−) mice develop a congenital nephrotic syndrome with podocyte foot-process effacements and die at 6 weeks of age from renal failure [34].

In the present study, we show for the first time that a reduced nephrin expression is not necessarily required for proteinuria, but also that enhanced nephrin protein levels may correlate with proteinuria as found in a rat model of mesangioproliferative glomerulonephritis. Total glomerular nephrin mRNA and protein expression per glomerulus constantly increased over 29 days and thereby paralleled the loss of renal function as indicated by increased protein concentrations found in the urine. However, by histological examinations, it is striking that not all podocytes show an increased fluorescence for nephrin but that some podocytes rather show a complete deficiency of nephrin. In total, the increase in nephrin per glomerulus dominates. An explanation for this intriguing observation can presently not be given but it may be speculated that both states, i.e., nephrin depletion and/or upregulation, disturb the stoichiometry of the normal filtration barrier complex. Clearly, an accumulation of nephrin in the slit diaphragm will also lead to altered protein–protein interactions and ultimately to dysregulated ultrafiltration.

However, it may also be possible that the upregulated de novo synthesis of nephrin is a protective mechanism by which the filtration machinery tries to compensate for an accelerated loss of protein. Previously, we have shown in cell culture systems of human primary podocytes and in the human embryonic kidney epithelial cell line A293 that proinflammatory cytokines like IL-1β and TNFα increase nephrin expression on a transcriptional as well as on the translational level [12]. In view of these cellular events, it seems obvious that in inflammatory kidney diseases like the mesangioproliferative glomerulonephritis, which display increased cytokine production [4,35], nephrin expression is upregulated.

Interestingly, Wernerson et al. [36] reported an ultrastructural redistribution of nephrin during a nephrotic syndrome from the area of foot processes into the cytoplasm of podocytes. Furthermore, the reduction of nephrin from the foot processes occurred irrespective of whether foot processes were intact or retracted, suggesting that the reduction of nephrin is not a phenomenon that is merely secondary to the effacement of foot processes. In view of these data, it would be interesting to investigate the subcellular localization of nephrin in podocytes during the course of THY1-GN.

The transmembrane protein nephrin interacts with various other proteins to build the filtration complex at the slit diaphragm. Additional members in this complex include podocin, Neph-1, CD2AP, and eventually other still to be identified proteins that have structural as well as signalling functions [37]. It is clear that a stoichiometric ratio between the proteins that form the filtration complex is essential for a normal functioning of ultrafiltration. A disturbance of this ratio either in one direction or the other may lead to a dysfunction of the barrier, in foot process flattening, and an uncontrolled passing of high-molecular weight proteins from the blood capillaries into the primary urine. Such a dysbalance may not only be triggered by changes in nephrin expression but also by changes in expressions of other members of the filtration slit complex. In this context, it is worth noting that in the course of anti-Thy1.1 nephritis we not only observed an upregulation of nephrin, but podocin and CD2AP were transiently downregulated and then normalized at the late phase of the disease although nephrin still remains elevated. Thus, the dysbalance is most prominent at early time points of the disease but persists up to day 29, the latest time point evaluated.

The mechanism by which pro-inflammatory cytokines upregulate nephrin expression is still unknown. Recently, the promoters of the human and the mouse nephrin genes have been cloned [38,39]. They contain various putative transcription factor recognition sites, including those for GATA-1, GATA-2, AP-2, AP-4, Ets1, NFAT, PAX2, C/EBP, SP1, and PPAR. It is tempting to speculate that pro-inflammatory cytokines can activate one or several of these transcription factors which then trigger nephrin gene transcription. It is well established that IL-1β and/or TNFα are able to induce synthesis of the transcription factor GATA-1 in megakaryocytic cells [40] as well as of AP-2 in renal mesangial cells [41], Ets-1 in rheumatoid arthritis synovial membranes [42] and in endothelial cells [43,44], C/EBP in human glioblastoma cells [45], HepG3 cells [46], and lung fibroblasts [47]. However, it still needs to be proven whether these transcription factor binding sites are functional in the nephrin promoter and whether pro-inflammatory cytokines do indeed activate the corresponding transcription factors in glomerular podocytes, which then may also identify novel ways of therapeutic approaches to proteinuric kidney diseases.

Acknowledgments

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