INTRODUCTION

Proteinuria is a hallmark of glomerular injury and a predictor for end-stage renal disease (1, 2). Current data indicate that disruptions of the glomerular filtration barrier play a central role in the pathogenesis of glomerular proteinuria (3–5). Within the glomerular filtration barrier, the podocyte slit diaphragm has been most extensively studied and is considered to be the key component in determining glomerular permselectivity (6). In line with this notion, proteinuric animal models can be induced by genetic knockdown of podocyte-related genes or selective injury to the podocyte slit diaphragm with agents such as puromycin aminonucleoside or adriamycin (7). By contrast, the anti-Thy1 glomerulonephritis is characterized by complement-dependent mesangiolysis at the outset and manifests substantial proteinuria during the course (8). The mechanisms underlying the development of proteinuria in this disease have not been fully elucidated.

Inflammatory cytokines and fibrogenic mediators have been shown to modulate podocyte motility and albumin permeability in vitro (9–13). And mesangial-derived cytokines such as tumor necrosis factor (TNF)-α or transforming growth factor (TGF)-β1 can inhibit the expression of nephrin and ezrin by podocytes and induce podocyte death and detachment (14, 15). These observations imply the existence of a potential mesangial cell-podocyte crosstalk (16–18), and raise the possibility that certain cytokines produced by activated mesangial cells in anti-Thy1 glomerulonephritis may act in a paracrine manner on adjacent podocytes, which then cause proteinuria via alterations of glomerular permselectivity. The objective of this study was to investigate...
the potential mechanisms whereby proteinuria developed in anti-Thy1 disease and the modulation of which by selective signal transduction inhibitors, including the known antiproteinuric agent pentoxifylline (PTX) (19–24).

**MATERIALS AND METHODS**

**Induction of Anti-Thy1 Glomerulonephritis and Experimental Design**

The study was carried out under a protocol approved by Institutional Animal Care and Use Committee of National Taiwan University and complied with standards delineated in the ARRIVE guidelines for animal research (25).

Male Wistar rats weighing 190–220 g were obtained from and housed at the animal center of our institute (temperature: 22 ± 2°C; humidity: 50 ± 20%) and fed with LabDiet® 5001 (LabDiet, St. Louis, MO, USA) with free access to drinking water. The animals were randomly divided into a control group of 15 rats and 4 nephritic groups with or without treatment of 18 rats each. The control rats (group A) received 0.2 mL of 1× phosphate-buffered saline (pH 7.4) on d 0. The nephritic rats (group B) received an intravenous injection of 250 μg of a mouse anti-rat Thy1 monoclonal antibody (Cedarlane, Burlington, ON, Canada) diluted in 0.9% saline (via tail veins) on d 0 and were treated with vehicle from d 1 to d 5. Groups C–E were treated with the same dose of anti-Thy1 antibody as group B, plus an IκB kinase inhibitor, IMD-0354 (10 mg/kg, Tocris Bioscience, Bristol, UK) (group C), or an activin receptor–like kinase (ALK) inhibitor, SB431542 (20 mg/kg, Tocris Bioscience) (group D), administered intraperitoneally daily from d 1 to 5, or PTX (20 mg/kg, Sanofi-aventis, Laval, Quebec, Canada) (group E), administered intravenously daily from day –2 to d 5. The doses of IMD-0354 and SB431542 administered to animals were determined and prepared according to references provided by the manufacturer, while the dose of PTX was determined according to our previous study, which also showed a greater antiproteinuric efficacy of the drug by administration before, as opposed to after, nephritis induction (8). All animals in groups A–E were anesthetized with a single intraperitoneal injection of 8 mg/kg xylazine and 80 mg/kg ketamine, and groups of three to six rats were sacrificed at the end of d 0, 1, 3, 5 or 7 (group A only).

**Measurement of Proteinuria and Urinary Nephrin Excretion**

Twenty-four–hour urine collections were taken at different time points, and protein concentrations were quantified by the Bradford method (Bio-Rad, Hercules, CA, USA). Separate sets of urine samples were collected, and nephrin concentrations were measured using a commercially available enzyme-linked immunosorbent assay kit (Exocell, Philadelphia, PA, USA), adjusted for creatinine levels determined by a standard colorimetric method (Jaffe rate reaction).

**Renal Histopathology and Electron Microscopy**

Kidney sections were fixed with 4% paraformaldehyde and embedded in paraffin. Three-micrometer-thick sections were stained with hematoxylin and eosin, or periodic acid–Schiff reagents and examined by light microscopy (Nikon Instruments, Nikon Corporation, Tokyo, Japan). For immunohistological staining, kidney samples were fixed with 4% parafomaldehyde and embedded in paraffin. Three-micrometer sections were deparaffinized and rehydrated. Sections were treated with 0.3% H2O2 in methanol for 15 min to quench endogenous peroxidase activity and boiled for 10 min in 0.1 mol/L citrate buffer to retrieve antigens. To detect activation of TNF-α and TGF-β signaling pathways, sections were incubated with anti–phospho-NF-κB p65 (Ser276) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti–phospho-Smad2/3 (Ser465/467) (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C, washed in 1× phosphate-buffered saline and incubated with ImmPRESS™ polymer reagent (Vector Laboratories Inc., Burlingame, CA, USA) for 30 min at room temperature. After washing, sections were incubated with the VECTOR NovaRED kit (Vector Laboratories) for 2–15 min to produce a brown red product. To detect podocyte-related proteins, sections were incubated with the following primary antibodies: anti-nephrin, anti-synaptopodin, anti-Wilms tumor (WT)-1 (Santa Cruz Biotechnology) or anti-podocin (Sigma-Aldrich) at 4°C overnight. After washing, sections were incubated with the fluoroaphore-conjugated secondary antibody and then mounted and subjected to fluorescence microscopy (Leica DMRa, Leica Microsystems, Wetzlar, Germany).

The number of podocytes per glomerulus was assayed by counting WT-1+–positive cells. The ratio of the immunofluorescent staining area to the total area of the glomerulus was calculated as the area positively stained for podocyte-related proteins (synaptopodin, nephrin, podocin) per glomerular area by automated computer analysis with Image-Pro plus 6.0 (Media Cybernetics, Rockville, MD, USA), based on the method reported elsewhere (26).

For electron microscopic examinations, small blocks of kidneys were fixed in 2.5% buffered glutaraldehyde, postfixed in 2% osmium tetroxide, dehydrated in graded ethanol and embedded in epoxy resin. Ultrathin sections (0.1-μm-thick) were stained with uranyl acetate and lead citrate and examined by an electron microscope (Hitachi H-7100, Tokyo, Japan).

**Quantitative Polymerase Chain Reaction (PCR)**

The gene expression of isolated glomeruli was analyzed as follows. Complementary DNA was generated from glomerular total RNA using an iScript™ cDNA Synthesis Kit (Bio-Rad). The abundance of miRNAs for TNF-α, interleukin (IL)-1β, monocyte chemotactrant protein (MCP)-1, TGF-β1, TGF-β2, TGF-β3, activin-βA and activin-βB, as well as various filtration barrier proteins, were quantified by using the Bio-Rad MyiQ™

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single color real-time PCR detection system. The specific primer pairs used are listed in Supplementary Table S1. Briefly, 1 μL of the cDNA was mixed with 200 nmol/L of primers and 1× Bio-Rad iQ™ SYBR Green Supermix. The gene expression levels of all cDNA samples were normalized by using 18S rRNA. The mean levels of the target gene/18S rRNA gene in normal glomeruli were arbitrarily defined as 1.0, and quantitative data were shown as the relative increase/decrease from control glomeruli.

**Western Blot Analysis**

Isolated glomeruli, before and after treatment with IMD-0354, SB431542 or PTX to the experimental animals, were lysed on ice in radioimmunoprecipitation assay buffer that contained 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate and 1 μg/mL each of aprotinin, leupeptin and pepstatin. Lysates were centrifuged at 17,608 g, and supernatants (50 μg protein/lane) were separated by 9% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto an Immobilon filter (Millipore, Bedford, MA, USA), as described previously (27). Temporal changes of α-smooth muscle actin (SMA) (Sigma-Aldrich), nuclear factor (NF)-κB p65, phospho-Smad2/3, podocyte-related proteins (nephrin, podocin, synaptopodin, WT-1 and zonula occludens [ZO]-1) (Santa Cruz Biotechnology), phospho-NF-κB p65 and Smad2/3 (Cell Signaling Technology) were then detected according to the manufacturers’ instructions.

**Statistical Analysis**

Data were expressed as mean ± standard error of the mean (SEM). One-way analysis of variance was performed by using PASW Statistics for Windows, version 18.0.0 (SPSS [IBM, Armonk, NY, USA]) to detect any differences among different groups of experimental animals. A $p$ value of <0.05 was considered statistically significant.

*All supplementary materials are available online at www.molmed.org.*

**RESULTS**

**Temporal Relationships between Cytokine Induction, Alteration of Filtration Barrier and Evolution of Proteinuria in Anti-Thy1 Glomerulonephritis**

After induction of the nephritis, proteinuria started to develop from d 1 and sustained to d 5 (Figure 1A). Appreciable amounts of nephrin were excreted in the urine during the same period (Figure 1B). On electron microscopy, mesangioytic changes within the mesangium were seen on d 1. Ultrastructural changes of the podocytes and endothelial cells were noted at different time points of the disease (Figure 1C). In parallel to these findings, quantitative PCR showed upregulation of glomerular mRNAs for inflammatory cytokines (TNF-α, IL-1β, and MCP-1) and fibrogenic molecules (TGF-β1, -2, and -3 and activin-βA), with TNF-α and
IL-1β prevailing in the early phase, and TGF-β and activin-βA dominating in the later stage (Figure 2A). Immunoblotting results demonstrated induction of p-NF-κB p65 earlier than that of p-Smad2/3 within the glomeruli (Figure 2B).

Quantitative PCR showed significant downregulation of glomerular mRNAs for podocyte-related nephrin, podocin, synaptopodin, CD2-associated protein (CD2AP) and endothelium-derived syndecan-4 and glipican-1 after induction of the nephritis. We also observed upregulation of mRNAs for hyaluronidase and heparanase, but not agrin or perlecan, at some points of the disease (Figure 3A). Immunoblotting results showed downregulation of glomerular WT-1, nephrin, podocin and synaptopodin from the outset, and induction of glomerular α-SMA from d 3, which coincided temporally with rebound proliferation of mesangial cells (Figure 3B).

Effects of IκB Kinase Inhibition on Proteinuria in Anti-Thy1 Glomerulonephritis

Treatment with an IκB kinase inhibitor, IMD-0354, resulted in attenuation of proteinuria on d 1, but not d 3 or 5, of the nephritis (Figure 4A). IMD-0354 attenuated the activation of NF-κB p65 within the glomeruli on d 1 (Supplementary Figure S1A) and partially restored the downregulated glomerular nephrin, synaptopodin and CD2AP mRNAs (Supplementary Figure S1B) as well as the downregulated glomerular WT-1, nephrin and synaptopodin proteins (Supplementary Figure S1C).

Effects of Activin Receptor–Like Kinase Inhibition on Proteinuria in Anti-Thy1 Glomerulonephritis

Treatment with an ALK inhibitor, SB431542, led to attenuation of proteinuria on d 3 and 5, but not d 1, of the nephritis (Figure 4B). SB431542 attenuated the activation of Smad2/3 within the glomeruli on d 5 (Supplementary Figure S2A) and partially restored the downregulated glomerular nephrin, synaptopodin, CD2AP and podocin mRNAs (Supplementary Figure S2B) as well as the downregulated glomerular WT-1, nephrin, podocin and synaptopodin proteins (Supplementary Figure S2C).

Mechanisms Whereby PTX Reduces Proteinuria in Anti-Thy1 Glomerulonephritis

The above results revealed that blocking the inflammatory or fibrotic pathway could attenuate glomerular injury at different time points. We then examined whether PTX, an agent capable of inhibiting both pathways (20,27,28), might exert a more complete control of proteinuria. Our results showed that treatment with PTX attenuated proteinuria (Figure 4C) and nephrinuria (Figure 5A) through the course of the disease. The treatment partially reversed...
the downregulated expression of podocyte-related mRNAs and proteins on d 1 (except podocin) and d 5 of the disease (Figures 5B, C). More importantly, the activation of glomerular NF-κB p65 and Smad2/3 was attenuated by PTX treatment on d 1 and 5, respectively (Figure 6).

DISCUSSION

Anti-Thy1 glomerulonephritis is a well-established nephritis model that simulates human mesangial proliferative glomerulonephritis. It is characterized by glomerular injury affecting primarily the mesangium yet displaying substantial proteinuria during the course. The precise mechanism of proteinuria in this disease has not been definitively established. One possibility is secondary podocyte damage due to physical stretching resulted from mesangial cell destruction, destabilization of capillary structure and formation of intraglomerular microaneurysms (29). This idea can be exemplified by an early study that showed occurrence of transient proteinuria in parallel with morphological changes of the podocyte and foot processes during the initial stage of the disease (30). Schaefer et al. (31) reported alterations in the expression of glomerular nephrin, podocin and CD2AP and suggested dysregulation of slit-diaphragm as a cause for proteinuria. Besides, we demonstrated ultrastructural changes of the filtration barrier structure, including alterations of the podocyte foot processes and podocytes as well as disrupted endothelium, irregularly thickened glomerular basement membrane and detached podocytes at different time points of the nephritis. In addition to physical forces, an alternative mechanism for proteinuria could be attributable to humoral factors that are induced during the disease. For example, inflammatory cytokines or fibrogenic mediators had been shown to modulate podocyte motility and albumin permeability, mRNA expression of podocyte markers such as nephrin and ezrin as well as podocyte survival and adhesion (9–15). Presumably, through mesangial cell-podocyte communication (16–18), these effects might lead to breakdown of the glomerular permeability with resultant formation of proteinuria.

In this study, we observed downregulation of glomerular nephrin, podocin, CD2AP and synaptopodin mRNAs in parallel with induction of TNF-α and IL-1β mRNAs on d 1. These data suggest a role of proinflammatory cytokines in the development of proteinuria during the early phase of the nephritis. In support of this hypothesis, the administration of an IκB kinase inhibitor, IMD-0354, to nephritic rats resulted in reduction of...
proteinuria and attenuation of glomerular p-NF-κB signals on d 1. Furthermore, the downregulated nephrin, synaptopodin and CD2AP mRNAs and WT-1, nephrin and synaptopodin proteins were restored partially by IMD-0354, indicating a possible pathogenetic role of TGF-β/IL-1/β/NF-κB signaling in proteinuria formation. IMD-0354 exerted its antiproteinuric effect only in the early phase but not at a later time. The reason for this result was not clear, but could be due in part to the rapid up-and-down expression of TGF-β1 and IL-1/β mRNAs, and activation of NF-κB p65. We surmise that mechanisms other than the TGF-β/IL-1/β/NF-κB cascade might be involved more substantially in proteinuria after d 3, which could not be modulated by treatment with IMD-0354.

The TGF-β/activin family for which expression prevailed in the mid to late phase of the nephritis may be a plausible candidate underlying proteinuria during that period. Apart from its profibrotic action, TGF-β1 induces podocyte dedifferentiation and causes albumin influx across the podocyte monolayer (11,12). In TGF-β1 transgenic mice, podocytes undergo apoptosis in association with depletion of podocytes early during progressive glomerulosclerosis (32). Furthermore, TGF-β1 overexpression in glomeruli has been shown to induce proteinuria along with effacement of the podocyte foot process and downregulation of nephrin and synaptopodin (33).

Similarly, activin A is an activator of renal glomerular and interstitial fibrosis (34,35). Nevertheless, compared with TGF-β1, relatively little information is available concerning the link between activin and proteinuria formation. In this study, we observed downregulation of glomerular nephrin, podocin and synaptopodin mRNAs and proteins that coincided temporally with induction of TGF-β1, TGF-β3 and activin-βA mRNAs during the mid and late phase of the nephritis. These temporal associations implicate a role for TGF-β/activin pathways in the pathogenesis of proteinuria in anti-Thy1 disease.

An early study evaluating the pathogenic role of Smad3 signaling in proteinuria formation demonstrated attenuation of streptozotocin-induced albuminuria in Smad3 knockout mice (36). In a subsequent report, TGF-β1 transgenic mice deficient for Smad3 exhibited less podocyte apoptosis, whereas mice heterozygous for CD2AP displayed reduced PI3K/Akt signaling as well as more severe podocyte apoptosis and proteinuria (37). These observations suggest that loss of CD2AP expression and/or function in podocytes may sensitize these cells to TGF-β/Smad3-dependent apoptosis, thereby contributing to proteinuria formation. In that context, the suppressed glomerular CD2AP mRNA and concomitant increased glomerular TGF-β1 and TGF-β3 mRNAs might act cooperatively to potentiate the pathogenic role of TGF-β3-Smad3 signaling in proteinuria during the later phase of anti-Thy1 glomerulonephritis.

By using pharmacological inhibitors, Grygielko et al. (38) showed that SB525334, a selective ALK5 inhibitor, reduced proteinuria in adriamycin-induced nephrosis in rats. ALK inhibitors act by suppression of TGF-β/Smad signals and inhibition of autoinduction of TGF-β and possibly activin βA (39). In line with these findings, we found that administration of SB431542, a prototypic ALK inhibitor, to rats with anti-Thy1 disease reduced proteinuria in association with attenuation of glomerular p-Smad2/3 signals and preservation of podocyte filtration barrier proteins (WT-1, nephrin, synaptopodin, podocin) during mid- to late-stage nephritis. These findings coincide with earlier reports showing similar inhibitory effects of proteinuria with distinct anti–TGF-β strategies in the same model (40–42).

Treatment with SB431542 to nephritic rats, however, did not attenuate proteinuria in the early phase. This result could be due to a lack of significant induction of TGF-β at the outset of the disease, implying an involvement of other pathways in proteinuria formation during...
the early phase. In support of this notion, a recent study showed that inhibiting TGF-β with soluble TGF-β-receptor anti-

body prevented fibrotic responses, but not proteinuria in murine adriamycin nephropathy (43). Given the differential

induction patterns of cytokines, and the insufficiency of either SB431542 or IMD-0354 to attenuate proteinuria through the entire course, it is tempting to speculate that agents capable of blocking multiple pathogenic pathways (for example, TNF-α, IL-1β and TGF-β/activins) may be required to achieve more complete control of proteinuria.

PTX is a clinically available phosphodiesterase inhibitor that has been shown to reduce proteinuria in various human kidney diseases, including mesangial proliferative glomerulonephritis and IgA nephropathy (19–24). The antiproteinuric effect of this drug is largely ascribed to its inhibitory action on TNF-α and/or TGF-β pathways (20,27,28). In agreement with these reports, the present study shows that PTX can reduce proteinuria in association with attenuation of nephrinuria, downregulation of glomerular NF-κB and Smad signals and restoration of podocyte-related filtration barrier proteins. The possibility of nonspecific interference with proteinuria by administration of PTX before the induction of nephritis was excluded in our previous study (8). However, it should be pointed out that the reversal of downregulated nephrin and podocin mRNA/protein by PTX was not complete, which likely accounted for residual proteinuria and nephrinuria despite treatment. In addition, alterations in other elements of the glomerular filtration barrier, such as the endothelial syndecan-4 and glypican-1, and the glycosaminoglycan degrading hyaluronidase and heparanase, have been shown to participate in the development of proteinuria (5,44–46). Because transcriptional regulation of these genes was not likely TNF-α- or TGF-β-dependent (47,48), PTX might not be able to modulate the dysregulated mRNA expression as seen in the present model.

CONCLUSION

Our data indicate that traditional pro-inflammatory and fibrogenic cytokines, such as TNF-α and TGF-β/activin, participate in the pathogenesis of protein-
Figure 6. Representative immunohistochemical staining showing p-NF-κB p65 (A) and p-Smad2/3 signals (B) on d 1 and 5, respectively (a, b and c denote control rats, vehicle-treated nephritic rats and PTX-treated nephritic rats, respectively; original magnification 400x). Bar graphs show semiquantitative results of p-p65–positive and p-Smad2/3–positive cells per glomerular cross-section. Values are the mean ± SEM of 25–30 glomeruli. **p < 0.01 versus control rats; #p < 0.05 versus vehicle-treated nephritic rats.

**ACKNOWLEDGMENTS**

We are grateful to professor Kuo-Shyan Lu and his members for technical support in the use of electron microscopy at the Department of Anatomy and Cell Biology, College of Medicine, National Taiwan University. This project was supported by grants from the National Science Council, Executive Yuan (grant nos. 96-2314-B-002-059-MY3, 99-2628-B-002-011-MY2), National Taiwan University Hospital (grant no. 100-SI568), the Ta-Tung Kidney Foundation and the Mrs. Hsiu-Chin Lee Kidney Research Fund, Taipei, Taiwan.

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