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ARTICLE *in* JOURNAL OF PROTEOME RESEARCH · DECEMBER 2006

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Serial Changes in Urinary Proteome Profile of Membranous Nephropathy: Implications for Pathophysiology and Biomarker Discovery

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Received March 28, 2006

Membranous nephropathy is one of the most common causes of primary glomerular diseases worldwide. The present study adopted a gel-based proteomics approach to better understand the pathophysiology and define biomarker candidates of human membranous nephropathy using an animal model of passive Heymann nephritis (PHN). Clinical characteristics of Sprague−Dawley rats injected with rabbit anti-Fx1A antiserum mimicked those of human membranous nephropathy. Serial urine samples were collected at Days 0, 10, 20, 30, 40, and 50 after the injection with anti-Fx1A (number of rats = 6; total number of gels = 36). Urinary proteome profiles were examined using 2D-PAGE and SYPRO Ruby staining. Quantitative intensity analysis and ANOVA with Tukey post-hoc multiple comparisons revealed 37 differentially expressed proteins among 6 different time-points. These altered proteins were successfully identified by MALDI-TOF MS and classified into 6 categories: (i) proteins with decreased urinary excretion during PHN; (ii) proteins with increased urinary excretion during PHN; (iii) proteins with increased urinary excretion during PHN, but which finally returned to basal levels; (iv) proteins with increased urinary excretion during PHN, but which finally declined below basal levels; (v) proteins with undetectable levels in the urine during PHN; and (vi) proteins that were detectable in the urine only during PHN. Most of these altered proteins have functional significance in signaling pathways, glomerular trafficking, and controlling the glomerular permeability. The ones in categories (v) and (vi) may serve as biomarkers for detecting or monitoring membranous nephropathy. After normalization of the data with 24-h urine creatinine excretion, changes in 34 of initially 37 differentially expressed proteins remained statistically significant. These data underscore the significant impact of urinary proteomics in unraveling disease pathophysiology and biomarker discovery.

Keywords: Heymann nephritis • urine • kidney • glomerulus • proteomics • proteome • biomarker • pathophysiology

Introduction

Membranous nephropathy, an idiopathic antibody-mediated autoimmune disease, is one of the most common causes of nephrotic syndrome in Caucasian adults.[1] Approximately one-third of affected patients will develop end-stage renal disease (or chronic renal failure) and eventually require renal replacement therapy.[2,3] Subepithelial immune complexes and complement activation lead to glomerular dysfunction and impairment of the filtration barrier.[4,5] A primary clinical manifestation is proteinuria, which is nonspecific to membranous nephropathy. At present, there is no reliable noninvasive method for predicting and/or monitoring this glomerular disease. Urine is an ideal source of materials to search for potential disease-related biomarkers as it is produced by the affected tissues and can be obtained easily by noninvasive methods. Therefore, the availability of urinary biomarkers that would provide diagnosis and/or prognosis of membranous nephropathy would be a significant advance. The characterization of urinary proteins may also lead to identification of new therapeutic targets and to better understanding of the disease pathophysiology.

In the present study, we adopted a gel-based proteomics approach to examine the urinary proteome profile in an animal model of human membranous nephropathy. Male Sprague−Dawley (SD) rats were injected with anti-Fx1A to induce passive Heymann nephritis (PHN), of which the characteristics truly

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Published on Web 10/05/2006
10.1021/pr060122b CCC: $33.50

resemble those of human membranous glomerulopathy. These include subepithelial immune deposits and severe proteinuria without cellular infiltration or inflammatory change in the glomeruli.[1,3] Serial urine samples were collected at baseline (Day 0), 10, 20, 30, 40, and 50 days following the induction of PHN to the animals. Urinary proteins were isolated using a precipitation method and separated with two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Resolved protein spots were visualized using SYPRO Ruby stain. Quantitative intensity analysis and ANOVA with Tukey post-hoc multiple comparisons were performed to define differentially expressed proteins, which were subsequently identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), followed by peptide mass fingerprinting using the NCBI protein database. A total of 37 proteins were identified as differentially expressed proteins. Their functional significance and potential roles in the pathophysiology of membranous nephropathy are discussed. Some proteins, which were undetectable or present only during the disease course, may be of further use as diagnostic or prognostic biomarkers.

Materials and Methods

Animals. Young male Sprague−Dawley (SD) rats (initial body weights = 180−200 g; $n = 6$ for each group; total $n = 12$) were used in this study. Animal care and treatment were conducted according to guidelines of the Department of Health, the Government of Hong Kong, SAR. All animals were housed in a room, in which the temperature was kept constant in a 12-h dark/12-h light cycle with ad libitum standard laboratory rat chow and tap water.

Induction of Passive Heymann Nephritis (PHN) Model. PHN was induced in 6 young male SD rats by intravenous injection of 1 mL rabbit anti-Fx1A antiserum. Fx1A was prepared from freshly isolated kidneys from normal SD rats (weight = 300−350 g) using standard protocol[6] and was utilized to immunize rabbits. Further 6 young male SD rats, with comparable body weights, were used as normal controls and were injected with an equal amount of normal rabbit serum. The efficacy of anti-Fx1A antiserum to induce PHN was tested by measuring urinary protein excretion and by renal histopathological and immunofluorescence examinations.

Renal Histopathological and Immunological Examinations. For light microscopic examination, snap-frozen kidneys of control and PHN rats at 14 days after injection (D14) with normal serum and anti-Fx1A antiserum, respectively, were cut into 5-μm-thick sections and stained with hematoxylin and eosin using a standard technique. For immunofluorescence examination, fresh tissues were embedded in OCT compound (Lab-Tek Products, Miles Laboratories, Naperville, IL) and snap-frozen in liquid nitrogen. Cryostat sections (5 μm thick) of D14 control and PHN kidneys were transferred to Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA). Tissue sections were fixed in acetone at 4 °C for 20 min and blocked with 20% horse serum in PBS for 60 min. Sections were then washed with cold PBS and stained with goat anti-rat IgG (1:200) conjugated with FITC for 1 h. Immunofluorescence was viewed with a confocal laser scanning microscope (Leica DMRBE, Solms, Germany). All images were captured using the Leica DC Viewer Digital Imaging Systems software package, using identical settings for exposure time and gain.

Urine Collection and Sample Preparation. The animals were transferred to metabolic cages individually and received free access of water but without food (to prevent contamination of proteins from food particles into the urine).[7,8] For total protein measurement, 24-h urine samples were collected on Day 0 and on Days 7, 14, 21, 28, 35, and 42 after anti-Fx1A antiserum injection. For proteomic analysis, 4-h urine samples were collected on Days 0, 10, 20, 30, 40, and 50. To minimize protein degradation, the urine samples for proteomic analysis were collected in 1 mL of protease-inhibitors cocktail (0.1 mg/mL leupeptin, 0.1 mg/mL phenylmethylsulfonyl fluoride, and 1 mM sodium azide in 1 M Tris, pH 6.8) in iceboxes.[7,8] Immediately after collection, the urine samples were centrifuged at 1000 × g for 5 min. After removal of cell debris and nuclei, proteins in supernatant were precipitated with 20% trichloroacetic acid in acetone containing 20 mM dithiothreitol (DTT) at −20 °C overnight followed by three washes with acetone/20 mM DTT. The pellets were centrifuged at 12 000 × g for 5 min and resuspended in a buffer containing 7.92 M urea, 0.06% SDS, 120 mM DTT, 3.2% Triton X-100, 22.4 mM Tris-HCl, and 17.6 mM Tris base (pH 8). Protein concentration of each sample was measured by spectrophotometry using Bio-Rad protein microassay based on the method of Bradford.[9]

2D-PAGE. A fixed amount of 200 μg of protein was taken from each sample for 2D-PAGE. The protein solution was then mixed with rehydration buffer containing 7 M urea, 2 M thiourea, 2% (w/v) 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate (CHAPS), 2% (v/v) ampholytes (pH 3−10), 120 mM DTT, 40 mM Tris-base, and bromophenol blue before loading onto an immobilized pH gradient (IPG) strip (linear pH gradient 3 to 10, 7-cm long; Amersham Biosciences, Uppsala, Sweden).[7,8] The samples were electrofocused to reach 9084 V·hours. After the completion of isoelectric focusing, the IPG strips were incubated in a buffer containing 6 M urea, 50 mM Tris (pH 8.8), 30% v/v glycerol, and 2% SDS with 65 mM DTT for 15 min and subsequently with 260 mM iodoacetamide for another 15 min. The second dimensional separation was performed with 50 mA in 15% acrylamide gel. The resolved 2D spots were visualized with SYPRO Ruby fluorescent dye (Bio-Rad, Hercules, CA) according to the manufacturer's instruction.

Quantitative Intensity Analysis. Image Master 2D Platinum (Amersham Biosciences) software was used for matching and analyzing protein spots on 2-D gels. Parameters used for spot detection were (i) minimal area = 10 pixels; (ii) smooth factor = 2.0; and (iii) saliency = 2.0. A reference gel was created from an artificial gel combining all of the spots presenting in different gels into one image. The reference gel was then used for matching of corresponding protein spots between gels. Background subtraction was performed and the intensity volume of individual spot was normalized with total intensity volume (summation of the intensity volumes obtained from all spots in the same 2-D gel).

Normalization of Intensity Data with 24-h Urine Creatinine. Urine creatinine concentrations of individual samples (from individual animals at each time-point) were measured using a standard spectrophotometric protocol. Briefly, urine samples were added into picric acid and sodium hydroxide containing SDS. Then the samples were allowed to stand at room temperature for 10 min and the absorbance was measured by a UV−visible spectrophotometer at λ505 nm. The 24-h urine creatinine excretion was calculated using the measured creatinine concentrations and 24-h urine volume, and was then used for normalization of the intensity data from individual protein spots.

Statistical Analysis. ANOVA with Tukey post-hoc multiple comparisons using the SPSS software (version 10.0) were

Table 1. Total Urine Protein Excretion in Control and PHN Animals

	urine protein (mg/24h)[a]	
days	control	PHN
0	6.47 ± 1.11	6.29 ± 0.44
7	7.39 ± 0.33	62.43 ± 5.55[b,c]
14	9.72 ± 0.65	142.76 ± 11.71[b,c]
21	8.12 ± 0.88	231.62 ± 25.17[b,c]
28	9.70 ± 2.23	261.69 ± 59.19[b,c]
35	9.23 ± 1.58	293.47 ± 42.10[b,c]
42	10.69 ± 2.10	317.09 ± 32.60[b,c]

[a] Data are expressed as Mean ± SEM; $n = 6$ for each groups. [b] $p < 0.001$ versus controls on the same day. [c] $p < 0.001$ versus day 0 of the PHN animals.

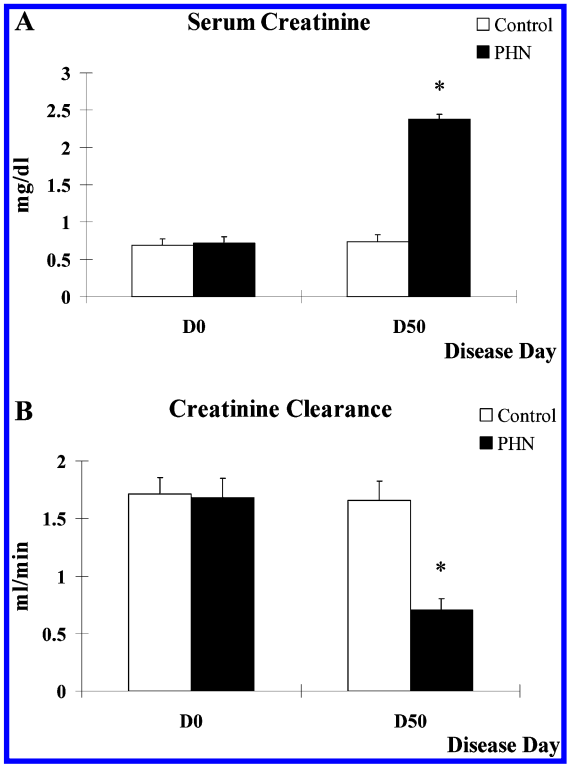

Figure 1. Serum creatinine level and creatinine clearance. (A) Rats had comparable serum creatinine levels at the baseline (D0). At D50, serum creatinine was increased significantly in the PHN rats compared to the controls. (B) Creatinine clearance, which represents renal function, significantly declined in the PHN rats. ($n = 6$ for each bar). * = $p < 0.001$; PHN vs controls.

performed to define significant differences among the different groups (time-points) of samples. *P* values less than 0.05 were considered statistically significant. A statistical power analysis was further conducted according to the method described by Hunt et al.[10] to ensure significant differences of the differentially expressed proteins among different time-points. The computational tool used to calculate such statistical power of significance was adapted from www.emphron.com. Setting of the statistical power was significance level of 5%, effect size of 100% and intensity changes of 2-fold, and the analysis was based on the experimental design of 6 individual rats at 6 different time-points.

MALDI-TOF MS and Peptide Mass Fingerprinting. Differentially expressed protein spots were subjected to identification using a reflectron MALDI-TOF mass spectrometer (Autoflex; Bruker Daltonics, Leipzig, Germany). These spots were excised and digested with trypsin according to the method described by Shevchenko et al.[11] The digests were desalted with ZipTip (Millipore, Boston, MA) and further analyzed by MALDI-TOF MS. Peptide mass fingerprinting was performed using ProFound search tool, which is available at http://129.85.19.192/ProFound_bin/WebProFound.exe, as well as MASCOT search engine, which is available at http://www/matrixscience.com, based on the entire NCBI protein database. The search was limited with a mass tolerance of 150 ppm. Only one missed cleavage *per* peptide was allowed and cysteine residues were assumed to be carbamidomethylated with acrylamide adducts and methionine residues were in oxidized form. Significant matching required Z score of >1.65 or probability-based MOWSE score of >78 ($p < 0.05$).

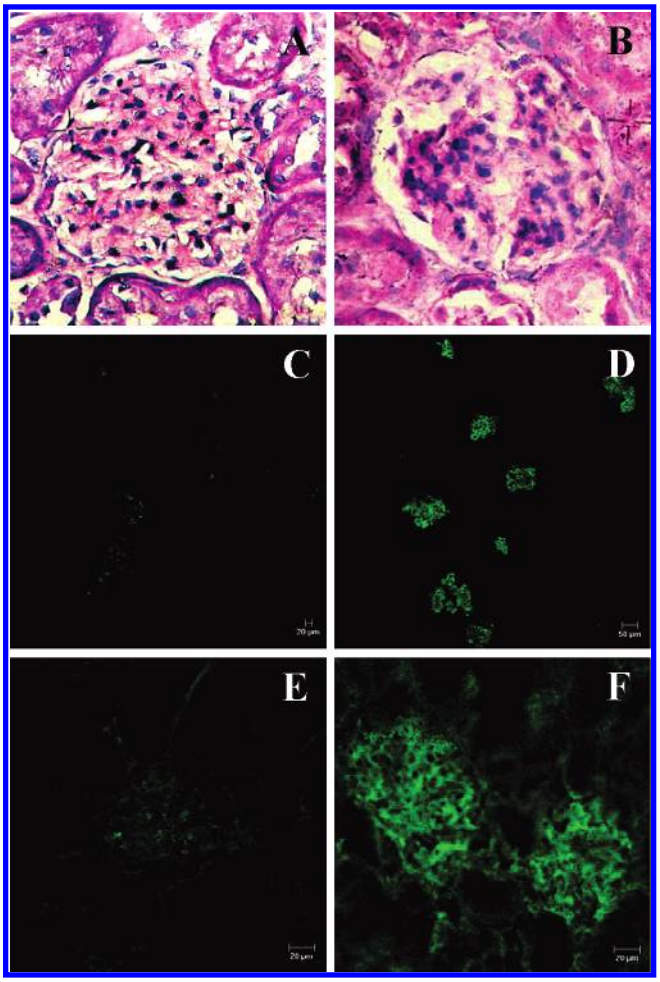

Figure 2. Histopathology and immunofluorescence study. Hematoxylin and eosin staining showed (A) normal glomerular structure of control rats and (B) the glomerulus with mesangial expansion and thickened glomerular basement membranes in PHN rats. Immunofluorescence study revealed tensed granular deposition of IgG in the glomeruli of PHN rats (D,F) that is typical for membranous nephropathy, whereas those in control rats had only weak background stain (C,E). Magnification powers were 400× for (A), (B), (E), and (F) and 100× for (C) and (D).

Results

24-h Urinary Protein Excretion. There were no significant differences of 24-h urine volumes between the controls and the PHN rats and among the different days of the disease (data not shown). But the total urine protein excreted over the 24-h

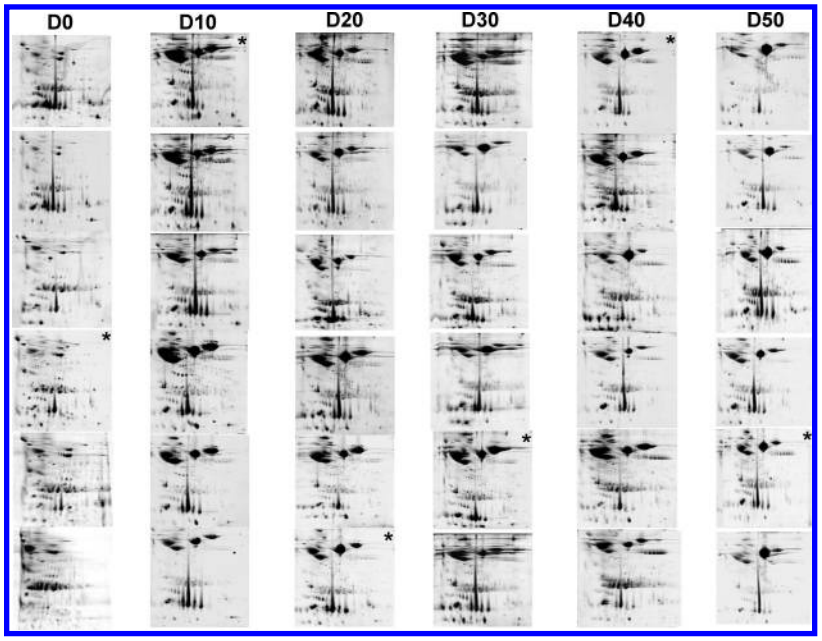

Figure 3. Urinary proteome profiles of PHN rats at different time-points of the disease. Urinary proteins were isolated using a precipitation method and resolved with 2D-PAGE using 7-cm-long, linear 3−10 pH gradient, IPG strips and 15% acrylamide gel. Resolved proteins were visualized with SYPRO Ruby stain. $N = 6$ gels from individual animals in each disease day. D0 = baseline, whereas D10−D50 = Day 10−Day 50. * = Represented gel series shown in Figure 4.

period in the PHN animals was significantly elevated ($p < 0.001$) throughout the disease course when compared to the controls at the same time-points and when compared to Day 0 (D0) of the PHN rats (Table 1). At the baseline (D0), rats in both groups (controls and PHN rats) had comparable urinary protein excretion levels (6.47 ± 1.11 and 6.29 ± 0.44 mg/24 h in controls and PHN rats, respectively). After 7 days (D7) of injection with rabbit anti-Fx1A antiserum to the PHN rats and normal rabbit serum to the controls, urinary protein levels of the control rats remained normal, whereas rats that received the anti-Fx1A antiserum developed significant proteinuria (7.39 ± 0.33 vs 62.43 ± 5.55 mg/24 h; controls vs PHN; $p < 0.001$; averaged PHN/controls ratio = 8.45). The PHN rats had progressive proteinuria throughout the study and urine protein levels at the end of experiment (D50) were 10.69 ± 2.10 mg/24 h in controls and 317.09 ± 32.60 mg/24 h in PHN rats ($p < 0.001$), and the averaged PHN/controls ratio was 29.65 (Table 1).

Serum Creatinine Level and Creatinine Clearance. Rats in both groups had comparable renal function at the baseline (D0). At D50, serum creatinine was increased significantly in the PHN rats compared to the controls (0.735 ± 0.097 vs 2.378 ± 0.071 mg/dl; controls vs PHN; $p < 0.001$) (Figure 1A). As a result, the creatinine clearance, which represents renal function, significantly declined in the PHN rats (1.657 ± 0.171 vs 0.703 ± 0.102 mL/min; controls vs PHN; $p < 0.001$) (Figure 1B).

Histopathological Findings. At D14, the glomeruli of control rats were normal (Figure 2A), whereas those of PHN rats had thickened glomerular basement membranes and increased mesangial matrix, while number of mesangial cells was not increased (Figures 2B). Immunofluorescence study showed extensive granular deposition of IgG in the glomeruli of PHN rats that is typical for membranous nephropathy[4] (Figure 2D,F).

Serial Changes of the Urinary Proteome Profile and Identification of Differentially Expressed Proteins. Serial collection of urine samples from 6 PHN rats was performed at D0, D10, D20, D30, D40, and D50 (days after the injection of anti-Fx1A antiserum) (total number of samples was 36). The contamination of proteins from food particles that might occur and interfere with the analysis was avoided by housing the rats in metabolic cages with free access of water but without food for 4 h.[7,8] The volume of urine collected from each animal over the 4-h period was comparable among different time-points (2.63 ± 0.31, 4.28 ± 0.82, 4.40 ± 0.87, 4.60 ± 0.89, 3.58 ± 0.33 and 3.82 ± 0.45 mL for D0, D10, D20, D30, D40, and D50, respectively, $p = 0.340$ by ANOVA). Cell, debris, and other particulate matters were discarded from the urine immediately after the collection. Urinary proteins were then concentrated from all these samples using a precipitation method. For qualitative analysis of the proteome profile and for quantitative intensity analysis to compare relative amounts of individual proteins among different time-points of the disease, the equal amount of total protein (200 μg) isolated from each urine sample was used for such comparisons. The isolated proteins were resolved with 2D-PAGE and visualized with SYPRO Ruby fluorescence dye. Figure 3 shows all 2D gels of individual urine samples ($n = 36$). A clear difference among groups (time-points) of samples can be observed.

Quantitative intensity analysis using a 2D analysis software (Image Master 2D Platinum; Amersham Bioscience) and ANOVA with Tukey post-hoc multiple comparisons revealed differential expression of 37 proteins among groups (time-points) (labeled in Figure 4). Power calculation revealed that the statistical power of the study is greater than 80% with the significance level of 5% and intensity changes of 2-fold. The differentially expressed proteins were then identified by MALDI-TOF MS (Table 2). Their expected p*I*, molecular size, sequence coverage (%Cov), Z score, probability-based MOWSE score, and quantitative intensity data are shown in Table 3. These proteins can be divided into 6 main categories: (i) proteins with decreased urinary excretion during PHN; (ii) proteins with increased urinary excretion during PHN; (iii) proteins with increased urinary excretion during PHN, but which finally

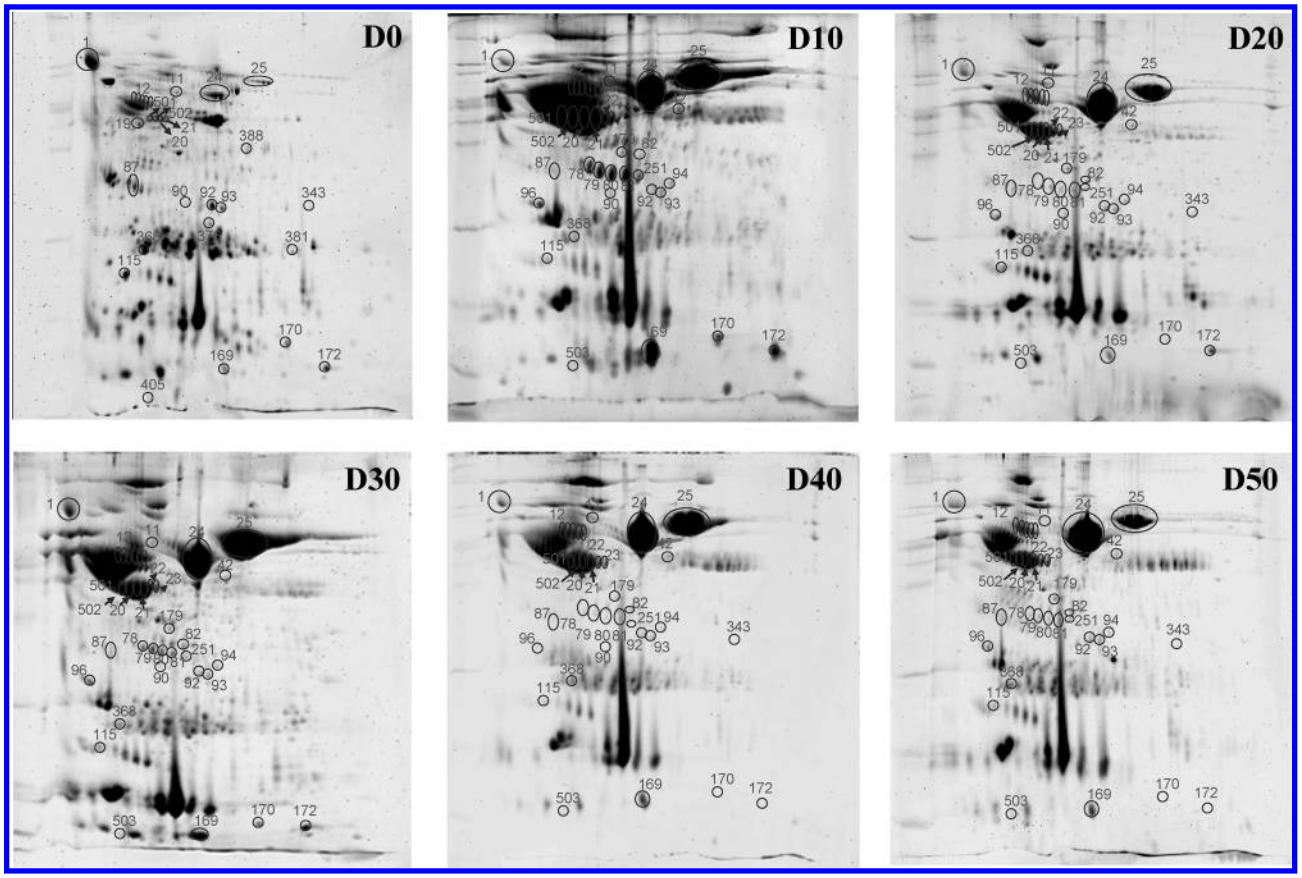

Figure 4. 2D map of differentially expressed proteins. Quantitative intensity analysis using Image Master 2D Platinum software (Amersham Bioscience) and ANOVA with Tukey post-hoc multiple comparisons revealed 37 differentially expressed proteins. All of these altered proteins were identified by MALDI-TOF MS and are labeled with numbers correspond to those in Tables 1 and 2. ($p < 0.05$, power > 80%)

returned to basal levels; (iv) proteins with increased urinary excretion during PHN, but which finally declined below basal levels; (v) proteins with undetectable levels in the urine during PHN; and (vi) proteins that were detectable in the urine only during PHN. Of all these differentially expressed proteins, the ones in categories (v) and (vi) may serve as biomarkers for detecting or monitoring membranous nephropathy.

Normalization of the Intensity Data with 24-h Urine Creatinine. To strengthen and standardize our data, we also normalized the intensity data with 24-h urine creatinine excretion. Table 4 shows the normalized quantitative data (x 10^{-3} arbitrary units per mg creatinine per 24 h). After normalization, changes in 34 of initially 37 differentially expressed proteins remained statistically significant by ANOVA and Tukey post-hoc multiple comparisons with the same pattern of changes as mentioned in Tables 2 and 3. The normalized data of the other 3 spots (#11, #20, and #251), although did not reach the statistically significant threshold, tended to be altered during different time-points. These consistent results strengthened our quantitative data.

Discussion

The diagnosis and treatment of glomerular diseases are presently based primarily on clinical manifestations, urinary protein excretion levels, and renal histopathology.[12] Inevitably, urinary protein excretion levels are not able to differentiate types of glomerular diseases. Renal biopsy, though providing specific diagnosis, is an invasive procedure with considerably significant complications, particularly in patients with bleeding tendency or skin infection on the flank. Thus, a renal biopsy may not be possible or contraindicated in certain high-risk patients. Serial renal biopsies, which can be used to monitor the outcome of treatment with immunosuppressant regimen, are frequently avoided because of the risk for serious complications. As the excretion levels of albumin and total protein can be affected by several factors (e.g., glomerular function, glomerular filtration rate, renal plasma flow, urine output, etc.), evaluation only for total protein or albumin excretion levels may be insufficient to monitor the therapeutic response or disease activity.[13] Defining a novel biomarker in the urine for each specific type of glomerular disease is therefore required and such biomarkers can serve as a noninvasive test for more effective prediction of the disease, earlier diagnosis, and better monitoring of the disease activity.

In the present study, we employed the passive Heymann nephritis (PHN) rat model to evaluate whether there is a set of urinary proteins that can be used as novel biomarkers for human membranous nephropathy. Clinical characteristics of the PHN rats mimicked those in human membranous nephropathy.[14] These included progressive proteinuria, declined renal function, mesangial expansion, thickened glomerular basement membrane, and granular deposition of IgG. The urinary protein and creatinine clearance data presented herein are consistent with those reported in previous study[15] that rats with PHN produce significant proteinuria and have impaired renal function. Hence, this animal model is suitable for such

Table 2. Differentially Expressed Proteins among Different Time-Points

	altered proteins	GenInfo ID	accession
I. Proteins with decreased excretion levels in the urine during PHN			
#1	epithelial-cadherin precursor (E-cadherin) (Uvomorulin) (Cadherin-1)	gi|13431333	Q9R0T4
#87	phosphatase subunit gene g4−1 [Mus musculus]	gi|19354389	AAH24754
#90	tropomyosin beta chain (Tropomyosin 2) (Beta-tropomyosin)	gi|20178269	P58775
#92	kallikrein 7; kallikrein 1 [Rattus norvegicus]	gi|6981132	NP_036725
#93	kallikrein 7; kallikrein 1 [Rattus norvegicus]	gi|6981132	NP_036725
#115	alpha-2u globulin	gi|204264	AAA41199
#343	aldehyde dehydrogenase family 1, subfamily A1 [Mus musculus]	gi|32484332	AAH54386
#368	Mpp7 protein [Mus musculus]	gi|29437038	AAH49662
II. Proteins with increased excretion levels in the urine during PHN			
#12	mKIAA1601 protein [Mus musculus]	gi|37360470	BAC98213.1
#20	alpha-1-antitrypsin; serine (or cysteine) proteinase inhibitor clade A (alpha-1 antiproteinase antitrypsin) member 1 [Rattus norvegicus]	gi|51036655	NP_071964
#21	alpha-1-antitrypsin; serine (or cysteine) proteinase inhibitor clade A (alpha-1 antiproteinase antitrypsin) member 1 [Rattus norvegicus]	gi|51036655	NP_071964
#24	serum albumin precursor	gi|113580	P02770
#25	serotransferrin precursor (Transferrin) (Siderophilin) (Beta-1-metal binding globulin)	gi|6175089	P12346
#501	1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase delta 1 (Phosphoinositide phospholipase C) (PLC-delta-1) (PLC−III)	gi|130228	P10688
#502	glycerol-3-phosphate dehydrogenase, mitochondrial precursor (GPD-M) (GPDH-M)	gi|2494650	Q64521
III. Proteins with increased excretion levels in the urine during PHN, but which finally returned to basal levels			
#11	embryonic growth-associated protein EGAP [Mus musculus]	gi|21539896	AAM52342
#169	chain D, Rat Transthyretin	gi|3212535	1GKE
IV. Proteins with increased excretion levels in the urine during PHN, but which finally declined below basal levels			
#170	pancreatitis associated protein III [Rattus norvegicus]	gi|463280	AAA41809
#172	pancreatitis associated protein III [Rattus norvegicus]	gi|463280	AAA41809
V. Proteins with undetectable excretion in the urine during PHN			
#19	progesterone-induced blocking factor 1 isoform a [Mus musculus]	gi|46852193	NP_083596
#376	unnamed protein product [Mus musculus]	gi|26353410	BAC40335
#381	ribosomal protein L5 [Rattus norvegicus]	gi|38014831	AAH60561
#388	vimentin [Rattus norvegicus]	gi|38197662	AAH61847
#405	similar to tropomyosin isoform 6 [Mus musculus]	gi|38083544	XP_355057
VI. Proteins that were detectable in the urine only during PHN			
#22	alpha-1-antitrypsin precursor	gi|203063	AAA40788
#23	alpha-1-antiproteinase precursor (Alpha-1-antitrypsin) (Alpha-1-proteinase inhibitor)	gi|112889	P17475
#42	serotransferrin precursor (Transferrin) (Siderophilin) (Beta-1-metal binding globulin)	gi|6175089	P12346
#78	ba1−647 [Rattus norvegicus]	gi|33086640	AAP92632
#79	haptoglobin precursor	gi|123513	P06866
#80	ba1−647 [Rattus norvegicus]	gi|33086640	AAP92632
#81	ba1−647 [Rattus norvegicus]	gi|33086640	AAP92632
#82	serum albumin precursor	gi|113580	P02770
#94	CTP:phosphocholine cytidylyltransferase	gi|455294	AAA53526
#96	similar to anti-idiotype immunoglobulin M light chain [Rattus norvegicus]	gi|34869770	XP_213585
#179	serum albumin precursor	gi|113580	P02770
#251	preprohaptoglobin	gi|204657	AAA41349
#503	vascular endothelial growth factor A splice variant VEGF 102 [Mus musculus]	gi|32699992	AAP86647

purpose. Moreover, the extensive study of urinary proteins in this animal model may also lead to better understanding of the disease pathophysiology in human membranous nephropathy.[14]

Gel-based proteomic methodology was applied to examine serial changes in the urinary proteome profile of PHN rats. To our knowledge, this is the first study that evaluates the urinary proteome at several different time-points, which directly refer to the disease course. The information obtained is more dynamic than studying only at a single time-point. We thus believe that this approach is most suitable for identifying biomarkers for disease monitoring. Using quantitative intensity analysis and appropriate statistical tests for multiple comparisons, a total of 37 protein spots were defined as differentially expressed proteins among groups, either normal (at the baseline or D0) vs disease (D10 to D50), or among the disease at differential stages (D10 to D50). These differentially expressed proteins were then successfully identified by MALDI-TOF MS and peptide mass fingerprinting. They could be classified into several categories; a part of them may be helpful for better understanding of the pathogenic mechanisms or pathophysiology of membranous nephropathy, whereas some of them may potentially serve as biomarker candidates. Functional significance as well as potential roles of some of these altered proteins in human membranous nephropathy is highlighted as follows.

A. Potential Biomarkers for Membranous Nephropathy. Theoretically, suitable protein biomarkers should be the ones whose expression levels are detectable only in the disease of interest or, *vice versa*, the ones whose expression was absent or undetectable in the disease. The findings in our present study showed that several proteins could potentially be useful as novel biomarkers for the early diagnosis of human membranous nephropathy. These include the proteins whose excretion was absent or undetectable during PHN (i.e., progesterone-induced blocking factor 1 isoform a, unnamed protein product, ribosomal protein L5, vimentin, and tropomyosin isoform 6), and those whose excretion levels were detectable only during PHN (i.e., alpha-1-antitrypsin, serotransferrin precursor, Ba1−647, haptoglobulin precursor, preprohaptoglobin, serum albumin precursor, CTP:phosphocholine cytidylyltransferase, similar to anti-idiotype immunoglobulin M light chain, and vascular

Table 3. Quantitative Data and Peptide Mass Fingerprint Scores[a]

spot no.	% Cov	p*I*	mw	Z score	MOWSE	intensity levels (mean ± SEM; arbitrary units) D0	D10	D20	D30	D40	D50
						Group I					
#1	14	4.7	99.21	2.33	44	2.556 ± 0.318	0.457 ± 0.135	0.723 ± 0.234	1.134 ± 0.314	0.960 ± 0.174	0.545 ± 0.129
#87	26	5.1	53.62	2.43	N/A[b]	0.349 ± 0.051	0.044 ± 0.017	0.098 ± 0.021	0.127 ± 0.050	0.094 ± 0.019	0.067 ± 0.014
#90	61	4.7	32.93	2.43	45	0.161 ± 0.074	0.041 ± 0.013	0.033 ± 0.011	0.020 ± 0.008	0.007 ± 0.003	0.000 ± 0.000
#92	29	5.6	29.52	2.21	60	0.416 ± 0.061	0.046 ± 0.006	0.068 ± 0.010	0.053 ± 0.009	0.055 ± 0.011	0.078 ± 0.016
#93	29	5.6	29.52	1.28	42	0.212 ± 0.037	0.057 ± 0.006	0.081 ± 0.008	0.057 ± 0.007	0.048 ± 0.011	0.079 ± 0.019
#115	62	5.4	17.34	2.38	92	0.802 ± 0.199	0.136 ± 0.046	0.075 ± 0.019	0.134 ± 0.026	0.083 ± 0.013	0.083 ± 0.023
#343	39	8.3	55.08	2.43	46	0.119 ± 0.040	0.000 ± 0.000	0.048 ± 0.012	0.000 ± 0.000	0.032 ± 0.021	0.011 ± 0.011
#368	57	6.6	40.70	2.43	66	1.856 ± 0.441	0.087 ± 0.015	0.162 ± 0.045	0.091 ± 0.020	0.258 ± 0.058	0.079 ± 0.017
						Group II					
#12	42	5.3	98.93	2.09	N/A[b]	0.283 ± 0.047	2.343 ± 0.341	2.844 ± 0.436	1.311 ± 0.237	2.955 ± 0.465	1.919 ± 0.229
#20	25	5.7	46.28	1.85	68	0.268 ± 0.017	1.970 ± 0.516	1.380 ± 0.151	1.467 ± 0.181	1.733 ± 0.292	1.788 ± 0.327
#21	25	5.7	46.28	2.17	74	0.180 ± 0.023	1.432 ± 0.302	1.135 ± 0.151	0.781 ± 0.102	1.013 ± 0.159	1.002 ± 0.220
#24	34	6.1	70.70	2.42	164	2.648 ± 0.503	9.361 ± 1.080	13.249 ± 1.522	14.071 ± 2.801	14.655 ± 1.627	28.160 ± 4.279
#25	51	7.0	78.57	2.43	132	1.373 ± 0.174	10.176 ± 0.879	9.830 ± 1.397	11.225 ± 1.604	10.955 ± 1.602	6.828 ± 0.580
#501	19	5.9	86.80	2.43	37	0.112 ± 0.011	1.292 ± 0.239	0.969 ± 0.087	1.127 ± 0.104	1.037 ± 0.254	1.005 ± 0.154
#502	53	6.2	81.40	2.43	N/A[b]	0.201 ± 0.037	1.179 ± 0.441	1.567 ± 0.192	1.571 ± 0.080	1.500 ± 0.317	1.447 ± 0.220
						Group III					
#11	54	6.9	84.36	2.43	40	0.017 (0.012	0.064 ± 0.011	0.076 ± 0.010	0.034 ± 0.014	0.024 ± 0.013	0.012 ± 0.004
#169	73	6.0	13.11	2.43	97	0.258 ± 0.047	1.735 ± 0.424	0.838 ± 0.314	1.100 ± 0.327	0.728 ± 0.137	0.247 ± 0.059
						Group IV					
#170	50	8.0	19.47	2.30	64	0.132 ± 0.038	0.187 ± 0.064	0.094 ± 0.015	0.064 ± 0.024	0.035 ± 0.011	0.021 ± 0.016
#172	54	8.0	19.47	2.35	75	0.796 ± 0.366	0.803 ± 0.222	0.303 ± 0.053	0.212 ± 0.051	0.114 ± 0.051	0.027 ± 0.016
						Group V					
#19	45	5.9	90.00	2.17	56	0.264 ± 0.073	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
#376	21	5.6	40.02	2.43	N/A[b]	0.176 ± 0.056	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
#381	17	9.9	34.67	2.43	N/A[b]	0.083 ± 0.025	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
#388	44	5.1	53.77	2.43	53	0.224 ± 0.062	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
#405	62	4.6	14.07	1.82	N/A[b]	0.207 ± 0.075	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
						Group VI					
#22	36	5.7	45.99	2.43	N/A[b]	0.000 ± 0.000	0.429 ± 0.031	0.311 ± 0.038	0.303 ± 0.031	0.308 ± 0.062	0.229 ± 0.029
#23	35	5.7	46.29	2.43	N/A[b]	0.000 ± 0.000	0.350 ± 0.055	0.259 ± 0.037	0.201 ± 0.034	0.176 ± 0.029	0.142 ± 0.014
#42	29	7.0	78.57	2.43	48	0.000 ± 0.000	0.051 ± 0.007	0.059 ± 0.007	0.040 ± 0.005	0.047 ± 0.010	0.016 ± 0.004
#78	26	6.1	43.08	2.40	76	0.000 ± 0.000	0.180 ± 0.066	0.046 ± 0.008	0.061 ± 0.024	0.038 ± 0.010	0.009 ± 0.007
#79	22	6.1	39.04	2.30	89	0.000 ± 0.000	0.281 ± 0.084	0.080 ± 0.011	0.094 ± 0.037	0.057 ± 0.012	0.017 ± 0.011
#80	22	6.1	43.08	2.35	54	0.000 ± 0.000	0.304 ± 0.100	0.092 ± 0.013	0.098 ± 0.035	0.043 ± 0.012	0.011 ± 0.008
#81	23	6.1	43.08	2.40	81	0.000 ± 0.000	0.527 ± 0.132	0.309 ± 0.051	0.149 ± 0.070	0.067 ± 0.015	0.043 ± 0.029
#82	27	6.1	70.70	2.17	72	0.000 ± 0.000	0.048 ± 0.007	0.053 ± 0.007	0.062 ± 0.008	0.052 ± 0.003	0.077 ± 0.028
#94	44	6.8	42.03	2.43	N/A[b]	0.000 ± 0.000	0.036 ± 0.009	0.049 ± 0.007	0.029 ± 0.002	0.034 ± 0.009	0.051 ± 0.019
#96	28	5.0	26.24	2.43	40	0.000 ± 0.000	0.089 ± 0.024	0.112 ± 0.017	0.091 ± 0.022	0.081 ± 0.028	0.049 ± 0.014
#179	21	6.1	70.70	2.43	56	0.000 ± 0.000	0.058 ± 0.008	0.066 ± 0.011	0.053 ± 0.018	0.032 ± 0.017	0.088 ± 0.028
#251	36	7.2	30.43	2.40	90	0.000 ± 0.000	0.085 ± 0.032	0.030 ± 0.005	0.025 ± 0.006	0.020 ± 0.005	0.041 ± 0.019
#503	38	4.9	15.00	2.43	32	0.000 ± 0.000	0.006 ± 0.004	0.040 ± 0.010	0.031 ± 0.015	0.013 ± 0.008	0.019 ± 0.007

[a] All spots had $p < 0.05$ by ANOVA with Tukey post-hoc multiple comparisons. [b] N/A = Not applicable (not appeared in the first 20 hits by MASCOT search, but had a very high Z score (>1.65) using the ProFound search tool).

endothelial growth factor A splice variant VEGF 102). However, the interpretation for the latter group (those with detectable levels only during PHN) must be used with caution because a few proteins, particularly albumin and transferrin, have several isoforms in the urine and some are readily detectable in the normal urine. The presentation of some isoforms of these two proteins only in the diseased urine, as in the present study, is possible and deserves further investigations for specificity or type of such isoforms, which are most likely modified by the processes called "post-translational modifications".

B. Proteins with Potential Roles in the Pathogenic Mechanisms or Pathophysiology of Membranous Nephropathy: Proteins with Decreased Excretion Levels in the Urine During PHN. Cadherins are type I membrane, calcium-dependent, cell adhesion molecules. These proteins preferentially interact with themselves and other molecules [e.g., catenins,[16] nephrin[17] which is a key component of podocyte slit diaphragm determining glomerular permeability] in a homophilic manner in connecting cells. Cell adhesive mechanisms have an impact on cell migration and proliferation, which are involved in the pathogenesis of various glomerular diseases. Down-regulation of cadherins, therefore, is associated with the glomerular permeability defects, thereby proteinuria. We identified a decrease in excretion of E-cadherin. Our data were consistent with the data reported previously of down-regulation of cadherins in several glomerular disorders.[18,19]

Tissue kallikrein is a serine proteinase that is involved in the production of the potent vasodilator, kinin peptide, from kininogen substrate.[20] Tissue kallikrein level is reduced in humans and animal models with hypertension, cardiovascular and renal diseases.[20,21] Another protein with a decreased excretion level during PHN is aldehyde dehydrogenase which is capable of converting 9-cis and all-trans retinal to corresponding retinoic acid with high efficiency.[22,23] It is strongly expressed in kidney, lung, testis, intestine, stomach, and trachea, and weakly in liver. Its function has been suggested to involve in oxidative pathways.[24] However, its role in glomerular disease has not been previously studied and further investigation may lead to the discovery of novel disease mechanisms for membranous nephropathy.

Table 4. Quantitative Data after Normalization with 24-h Urine Creatinine Excretion[a]

spot no.	normalized intensity levels (x 10^{-3})(mean ± SEM; arbitrary units/mg Cr/24 h)					
	D0	D10	D20	D30	D40	D50
			Group I			
#1	3.4776 ± 0.6191	0.2555 ± 0.0769	0.5881 ± 0.2006	0.6691 ± 0.0963	0.7796 ± 0.1573	0.3028 ± 0.0618
#87	0.4823 ± 0.1021	0.0258 ± 0.0099	0.0772 ± 0.0168	0.1068 ± 0.0613	0.0774 ± 0.0202	0.0382 ± 0.0073
#90	0.2154 ± 0.1016	0.0229 ± 0.0074	0.0270 ± 0.0087	0.0151 ± 0.0077	0.0058 ± 0.0030	0.0000 ± 0.0000
#92	0.5323 ± 0.0528	0.0267 ± 0.0046	0.0582 ± 0.0133	0.0351 ± 0.0052	0.0466 ± 0.0115	0.0477 ± 0.0116
#93	0.2651 ± 0.0308	0.0328 ± 0.0053	0.0657 ± 0.0095	0.0370 ± 0.0038	0.0418 ± 0.0114	0.0481 ± 0.0126
#115	0.9783 ± 0.1684	0.0781 ± 0.0269	0.0630 ± 0.0171	0.0870 ± 0.0147	0.0645 ± 0.0087	0.0460 ± 0.0120
#343	0.1494 ± 0.0453	0.0000 ± 0.0000	0.0422 ± 0.0117	0.0000 ± 0.0000	0.0245 ± 0.0161	0.0055 ± 0.0055
#368	2.3027 ± 0.4648	0.0532 ± 0.0129	0.1172 ± 0.0274	0.0533 ± 0.0122	0.2101 ± 0.0522	0.0451 ± 0.0091
			Group II			
#12	0.3939 ± 0.0926	1.3458 ± 0.2366	2.1488 ± 0.2228	0.9204 ± 0.2172	2.3434 ± 0.4214	1.1555 ± 0.1877
#20	0.3618 ± 0.0405	1.2269 ± 0.4393	1.1117 ± 0.1837	1.0700 ± 0.2880	1.3142 ± 0.1500	1.0754 ± 0.2165
#21	0.2458 ± 0.0459	0.8443 ± 0.2416	0.9524 ± 0.1925	0.5316 ± 0.0884	0.7943 ± 0.1169	0.5899 ± 0.1309
#24	3.4104 ± 0.4906	5.5143 ± 0.9054	10.8350 ± 1.8675	9.1490 ± 1.8234	12.0471 ± 2.0201	16.8190 ± 3.1887
#25	1.7627 ± 0.1073	5.9299 ± 0.8054	7.5727 ± 0.9350	7.8625 ± 1.7960	8.9028 ± 1.6294	4.0641 ± 0.4872
#501	0.1456 ± 0.0083	0.8030 ± 0.1965	0.7620 ± 0.0836	0.7901 ± 0.1691	0.8298 ± 0.2196	0.6060 ± 0.1156
#502	0.2715 ± 0.0634	0.7251 ± 0.3049	1.2143 ± 0.1455	1.0914 ± 0.1955	1.1824 ± 0.2578	0.8638 ± 0.1553
			Group III			
#11	0.0259 ± 0.0186	0.0379 ± 0.0080	0.0575 ± 0.0057	0.0277 ± 0.0152	0.0219 ± 0.0116	0.0073 ± 0.0028
#169	0.3262 ± 0.0389	1.0502 ± 0.3092	0.5928 ± 0.1587	0.8272 ± 0.2979	0.6163 ± 0.1236	0.1523 ± 0.0405
			Group IV			
#170	0.1783 ± 0.0567	0.1161 ± 0.0489	0.0762 ± 0.0153	0.0539 ± 0.0273	0.0298 ± 0.0096	0.0108 ± 0.0085
#172	1.0803 ± 0.4748	0.4429 ± 0.1217	0.2452 ± 0.0482	0.1532 ± 0.0525	0.0958 ± 0.0426	0.0143 ± 0.0089
			Group V			
#19	0.3274 ± 0.0732	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000
#376	0.2190 ± 0.0582	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000
#381	0.1123 ± 0.0354	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000
#388	0.2724 ± 0.0557	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000
#405	0.2418 ± 0.0712	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000
			Group VI			
#22	0.0000 ± 0.0000	0.2424 ± 0.0217	0.2482 ± 0.0312	0.2178 ± 0.0567	0.2582 ± 0.0659	0.1328 ± 0.0163
#23	0.0000 ± 0.0000	0.1945 ± 0.0258	0.2030 ± 0.0250	0.1459 ± 0.0403	0.1458 ± 0.0286	0.0831 ± 0.0092
#42	0.0000 ± 0.0000	0.0299 ± 0.0057	0.0449 ± 0.0039	0.0295 ± 0.0082	0.0377 ± 0.0087	0.0098 ± 0.0027
#78	0.0000 ± 0.0000	0.1101 ± 0.0485	0.0350 ± 0.0062	0.0506 ± 0.0270	0.0324 ± 0.0084	0.0057 ± 0.0044
#79	0.0000 ± 0.0000	0.1674 ± 0.0575	0.0614 ± 0.0072	0.0792 ± 0.0423	0.0479 ± 0.0116	0.0107 ± 0.0068
#80	0.0000 ± 0.0000	0.1811 ± 0.0685	0.0700 ± 0.0089	0.0786 ± 0.0367	0.0364 ± 0.0103	0.0071 ± 0.0050
#81	0.0000 ± 0.0000	0.3016 ± 0.0859	0.2542 ± 0.0499	0.1137 ± 0.0555	0.0564 ± 0.0117	0.0270 ± 0.0184
#82	0.0000 ± 0.0000	0.0268 ± 0.0035	0.0439 ± 0.0090	0.0407 ± 0.0057	0.0421 ± 0.0048	0.0488 ± 0.0199
#94	0.0000 ± 0.0000	0.0213 ± 0.0067	0.0413 ± 0.0099	0.0205 ± 0.0044	0.0300 ± 0.0090	0.0322 ± 0.0135
#96	0.0000 ± 0.0000	0.0532 ± 0.0180	0.0878 ± 0.0154	0.0634 ± 0.0179	0.0702 ± 0.0267	0.0299 ± 0.0098
#179	0.0000 ± 0.0000	0.0339 ± 0.0063	0.0561 ± 0.0147	0.0375 ± 0.0123	0.0294 ± 0.0167	0.0545 ± 0.0189
#251	0.0000 ± 0.0000	0.0507 ± 0.0219	0.0254 ± 0.0059	0.0197 ± 0.0083	0.0174 ± 0.0056	0.0264 ± 0.0132
#503	0.0000 ± 0.0000	0.0027 ± 0.0018	0.0341 ± 0.0093	0.0212 ± 0.0096	0.0098 ± 0.0069	0.0110 ± 0.0043

[a] Almost all spots (except only for #11, #20, and #251) had $p < 0.05$ by ANOVA with Tukey post-hoc multiple comparisons.

Proteins with Increased Excretion Levels in the Urine During PHN. Alpha-1-antitrypsin (AAT) is a member of the serine protease inhibitor (SERPIN) family. It is one of the most abundant proteins in urine of patients with proteinuria and has been suggested to be a marker for glomerulopathy.[18] Recently, another potential role of AAT has been suggested to involve in regulation of inflammation and mesangial matrix accumulation.[25,26] We identified the increase in urinary AAT excretion in our present study. This is consistent with the findings reported in previous studies.[18,25,26]

Vascular endothelial growth factor (VEGF; or vascular permeability factor) is a growth factor involved in angiogenesis, vasculogenesis, and endothelial cell growth. VEGF induces endothelial cell proliferation, promotes cell migration, inhibits apoptosis, enhances TGF-beta1 expression in mouse glomerular endothelial cells via mitogen-activated protein kinase and phosphatidylinositol 3-kinase, and induces permeabilization of blood vessels.[27] Our data are consistent with findings in several studies, which reported the up-regulation of VEGF in various glomerular diseases.[28−33] Another protein that plays a crucial role in protein kinase signaling pathways is 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase delta 1 (other synonyms are phosphoinositide phospholipase C, PLC-delta-1, and PLC−III), which mediates the production of the second messenger molecules diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3).[34] We identified the increase in excretion of PLC-delta-1 in a rat PHN model. These data suggest that VEGF and PLC-delta-1 are likely to play critical roles in glomerular trafficking, leading to glomerular injury in membranous nephropathy.

Among these altered proteins, E-cadherin and alpha-1-antitrypsin have been also proven to have the decreased and increased excretion levels, respectively, in human membranous nephropathy in patients with lupus nephritis class V.[18] Although kallikrein has not been previously proven to have a role in the pathogenic mechanisms of human membranous nephropathy, kininogen, which is another protein involved in the renal kininogen-kinin-kallikrein system (indeed, it is the substrate for bradykinin generation), has been already demonstrated to have an altered (decreased) excretion level in human membranous nephropathy.[18] These data strengthen our results and

indicate that proteomic data obtained from PHN animal model are applicable to human membranous nephropathy.

C. Intermediate Modulators for the Disease Processes that may Potentially Be Useful as Novel Biomarkers for Monitoring or Predicting the Disease Course. This group of proteins may have potential roles in pathogenic mechanisms of membranous nephropathy as the intermediate modulators for the disease processes; hence, their increase in expression did not last long and returned to the basal levels or even lower than the baselines. Interestingly, as changes in urinary excretion of these proteins were dynamic, these proteins may potentially be useful as novel biomarkers for monitoring or predicting the disease course. This group of proteins included embryonic growth-associated protein, pancreatitis-associated protein III, and transthyretin. Whereas the role for the former two proteins in glomerular diseases remains unclear, defects in transthyretin has been previously shown to be associated with glomerulopathy, particularly amyloidosis-related kidney disease.[35,36]

D. Altered Proteins whose Sequences Have been Recently Submitted to the Database but their Functions Remain Unknown. These include MPP7 [submitted in 2002[37]], mKIAA1601 protein [submitted in 2003[38]], unnamed protein product [submitted in 1999[39]], and Ba1−647 [direct submission in June 2003]. Functions or pathophysiological roles of these proteins remain unknown. Further investigations may lead to the discovery of novel pathogenic mechanisms of human membranous nephropathy.

Conclusions

Using a gel-based proteomic approach, we defined serial changes in urinary proteome profile of PHN rats. The altered proteins or those with differential urinary excretion levels during the disease course were successfully identified using mass spectrometry. Most of these 37 proteins have potential roles in the pathogenic mechanisms or pathophysiology of human membranous nephropathy, whereas some of them may potentially be novel biomarkers for early diagnosis, monitoring the disease course, and/or prediction of therapeutic outcome. With these preliminary data obtained from the animal model, our works on human membranous nephropathy will be highly focused as to further explore each individual protein in humans for future use in clinical practice.

Abbreviations: 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate; DTT, dithiothreitol; IPG, immobilized pH gradient; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PHN, passive Heymann nephritis; SD, Sprague−Dawley

Acknowledgment. We thank Professor R. Grimble for his editorial help and thank S. Chutipongtanate, N. Songtawee, T. Semangoen, and R. Kanlaya for their technical assistance. This study was supported by the Hong Kong Healthcare Association, Ltd, HK, the Matching Grant Scheme under the University Grants Committee of the Hong Kong Special Administration Region, China (Project No. 20600305) (to J.M.W.), and by Siriraj Grant for Research and Development (to V.T.).

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PR060122B