Urine Proteome Scans Uncover Total Urinary Protease, Prostaglandin D Synthase, Serum Amyloid P, and Superoxide Dismutase as Potential Markers of Lupus Nephritis

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Urine Proteome Scans Uncover Total Urinary Protease, Prostaglandin D Synthase, Serum Amyloid P, and Superoxide Dismutase as Potential Markers of Lupus Nephritis

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To identify potential biomarkers in immune-mediated nephritis, urine from mice subjected to an augmented passive model of anti-glomerular basement membrane (GBM)-induced experimental nephritis was resolved using two-dimensional gels. The urinary proteome in these diseased mice was comprised of at least 71 different proteins. Using orthogonal assays, several of these molecules, including serum amyloid P (SAP), PG D synthase, superoxide dismutase, rennin, and total protease were validated to be elevated in the urine and kidneys of mice during anti-GBM disease, as well as in mice with spontaneously arising lupus nephritis. Among these, urinary protease was the only marker that appeared to be exclusively renal in origin, whereas the others were partly serum-derived. Longitudinal studies in murine lupus demonstrated that total urinary protease had better predictive value for histologically active nephritis ($r = 0.78$) compared with proteinuria ($r = -0.04$), azotemia ($r = 0.28$), or the other markers examined, whereas urine SAP emerged as the single most predictive marker of histological glomerulonephritis. Collectively, these studies uncover total urinary protease, PG D synthase, SAP, and superoxide dismutase as novel biomarkers of anti-GBM disease and lupus nephritis, with stronger correlation to renal disease compared with currently employed biomarkers. These findings could have important diagnostic and prognostic ramifications in the management of these renal diatheses. The Journal of Immunology, 2010, 184: 2183–2193.

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease with diverse clinical presentations. Although virtually any organ can be involved, renal disease, which affects 25–50% of patients with lupus, is one of the leading causes of morbidity and mortality in this disease (1–4). Indeed, in various longitudinal studies, up to 50% or more of patients with lupus can develop renal flares over the follow-up period (3). Unfortunately, the clinical management of nephritis in this autoimmune disease is still an intricate problem (4, 5). Because early diagnosis and treatment of lupus nephritis is associated with better outcome, identifying specific biomarkers that can be used to diagnose disease, gauge its severity, and monitor response to treatment is of paramount importance (6–8). Currently, the presence of nephritis is gauged by measuring circulating and excreted indicators of renal dysfunction, with supporting information from renal biopsy. The measurement of excreted urinary protein or albumin appears to be the most reliable noninvasive method available for monitoring renal disease in lupus. Twenty-four-hour urine protein levels and albumin:creatinine ratios correlate well with each other and represent among the best biomarkers of renal disease currently available (9). Supplemented with readouts from urinalysis (e.g., cells/casts), serum creatinine levels, and renal biopsy information, the physician is able to plan an appropriate management strategy for the patient. Given that early detection and treatment of lupus nephritis is associated with better prognosis (6–8), there is an urgent need for better biomarkers of renal disease that one could potentially use to predict impending nephritis and monitor its progression in lupus.

Emerging biomarkers in lupus nephritis that have recently been reported include serum levels of various cytokines, mediators, or adhesion molecules (10–12), gene expression levels in urine cells (13–15), and urine levels of chemokines (16–19) and VCAM-1 (18–20). The latter two appear to be particularly promising because they can be assayed simply in voided urine, and they appear to correlate reasonably well with renal flares (17). Whereas earlier studies (16, 20) were designed based on the published properties of selected molecules (e.g., MCP-1 or VCAM-1), the current study is not biased by any preconceived notion based on published literature, but represents a comprehensive search for urinary molecules that may serve as potential biomarkers of nephritis.

A valuable tool for the study of lupus nephritis is experimentally induced renal disease following the administration of anti-glomerular basement membrane (GBM, or antiglomerular) Abs (21–23). This experimental model can be established in at least three different ways. In contrast to passive anti-GBM disease (where anti-GBM Abs alone induce disease upon transfer), anti-GBM disease accelerated by preimmunization with heterologous serum and anti-GBM disease accelerated by the coadministration of LPS are significantly more severe (21, 23). In contrast to spontaneous lupus nephritis, proteinuria, azotemia, and glomerular and tubulointerstitial disease all ensue with a rapid and predictable time course in the experimentally induced model (21–23). Over the past decade, researchers have assessed the roles of $>25$ different molecules (including various complement proteins and TLR...
ligands, FcR, B7/CD28/CTLA4, LFA1/ICAM1, P-selectin, TNF-α, IL-1, IL-6, IL-12, IL-18, IFN-γ, macrophage CSF, platelet-derived growth factor, MCP-1, and NO) in the pathogenesis of spontaneous lupus nephritis as well as experimental anti-GBM disease. Importantly, the molecules that have been studied thus far have shown excellent concordance in how they affect both disease settings, as recently reviewed (22). In other words, molecules known to influence the progression of experimental anti-GBM disease also impacted the development of spontaneous lupus nephritis in the same direction. Thus, although experimental anti-GBM nephritis and spontaneous lupus nephritis may differ in the nature of the initiating Abs and the localization of the immune deposits, a shared network of downstream molecular pathways appears to be mediating disease in both settings.

Indeed, our earlier studies have reaffirmed that selected molecules excreted in the urine during experimental anti-GBM disease are also present in the urine as a consequence of spontaneously arising lupus (18, 19). In the current study, we have executed an unbiased two-dimensional gel-based global search for proteins that may be hyperexpressed in the urine during experimental anti-GBM disease and then validated these findings in spontaneous lupus nephritis, both in mice and patients. Collectively, these studies have yielded a novel series of urinary molecules with good predictive value for Ab-mediated nephritis in mice and patients with lupus.

Materials and Methods

Mice and anti-GBM nephritis

C57BL/6 (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained in a specific pathogen-free colony. Females, aged 2–3 mo, were used for the anti-GMB–induced nephritis studies. The augmented passive model of anti-GBM nephritis was induced using a combination of LPS (50 μg, i.p.) and anti-GMBSera (240 μg, i.v.) administered as single injection on day 0, as described previously (23). Twenty-four-hour urine samples were collected from all mice on days 0, 3, 7, 10, 14, and 21 postinjection using metabolic cages, with free access to drinking water. Urinary protein concentration was determined using the Coomassie Plus protein assay kit (Pierce Chemical, Rockford, IL). The mice were sacrificed on day 21, and kidneys were procured for protein assays, as described below. In addition, 6-mo-old female MRL.lpr (24) and B6.Sle1.Sle2 (25) mice with spontaneous lupus nephritis were also studied.

Assessment of renal pathology

Renal tissues were processed for histology as detailed previously (18, 25). The glomerular and tubular histological disease scores were assessed by a blinded pathologist, as detailed previously (18). Briefly, the severity of glomerulonephritis (GN) was graded on a 0–4 scale, where 0 = normal; 1 = mild increase in mesangial cellularity and matrix; 2 = moderate increase in mesangial cellularity and matrix, with thickening of the GBM; 3 = focal endocapillary hypercellularity with obliteration of capillary lumina and a substantial increase in the thickness and irregularity of the GBM; and 4 = diffuse endocapillary hypercellularity, segmental necrosis, crescents, and hyalinized endstage glomeruli. The renal disease activity index is based on the evaluation of six histologic parameters (i.e., glomerular endocapillary proliferation, glomerular leukocyte infiltration, glomerular subendothelial hyaline deposits, glomerular fibrinoid necrosis or karyorrhexis, cellular crescents, and interstitial inflammation), each graded on a scale of 0–3, where 0 = absent; 1 = <25% glomeruli affected; 2 = 25–50% glomeruli affected; and 3 = >50% glomeruli affected. The scores for glomerular necrosis and cellular crescents are double-weighted due to their more ominous prognostic value. The sum (from 0–24) of each individual score represents the activity index. The renal disease chronicity index (from 0–12) was graded by summing the individual scores of four histologic features: glomerular sclerosis, fibrous crescents, tubular atrophy, and interstitial fibrosis.

Two-dimensional gel electrophoresis

Protein extraction buffer was composed of 13.3% TCA and 0.093% 2-ME in acetone. Three volumes of the chilled protein extraction buffer were added to urine samples and incubated overnight at −20°C. Mixtures were centrifuged for 15 min at 14,000 rpm, and the pellets were washed twice using chilled acetone containing 0.07% 2-ME to remove all TCA. The resulting proteins were solubilized in two-dimensional gel rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 100 mM DTT, 0.8% amphotelyte, and 0.02% bromophenol) at 30°C for 2 h. Eleven-centimeter-long Immobiline DryStrips (linear pH 4–7) (GE Healthcare, Fairfield, CT) were rehydrated overnight with 200 μg total protein in rehydration buffer, composed of 7 M urea, 2 M thiourea, 2% CHAPS, 2% amphotelytes (pH 3–10), 120 mM DTT, 40 mM Tris-base, and bromophenol blue, to make a final volume of 200 μl per strip. The first-dimensional isolectric focusing separation was performed using the Multiphor II system (GE Healthcare) for ~60 kV at 20°C. After completion of the isoelectric focusing, proteins on the strip were equilibrated with a buffer containing 7 M urea, 2% DTT, 50% glycerol, 100 mM Tris base, 4% SDS, and 0.002% bromophenol blue for 15 min, and then with a second buffer containing 7 M urea, 5% iodoacetamide, 30% glycerol, 100 mM Tris base, 4% SDS, and 0.002% bromophenol blue for 15 min. The strips were then transferred onto 12.5% Criterion gel (Bio-Rad, Hercules, CA), and the second-dimensional m.w.-based separation was performed using 20 mA/gel for 1.5 h. Separated protein spots were visualized using Sypro Ruby or colloidal blue (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Gel images were scanned using a Typhoon 9200 scanner (GE Healthcare) and analyzed using ImageMaster Platinum (GE Healthcare). Spots were excised, in-gel digested with trypsin, and injected into a reverse-phase nano HPLC/ion-trap mass spectrometer with nanospray source for sequencing.

Superoxide dismutase measurement

The renal cortex was separated from the kidney, homogenized in 7.5 ml cold 20 mM HEPES buffer (pH 7.2) containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose per gram of tissue, and centrifuged. Superoxide dismutase (SOD) in the supernatant was assayed using a colorimetric assay kit from Cayman Chemicals (Ann Arbor, MI). Briefly, 10 μl sample or standard was added to 200 μl diluted radical detector, and the reaction was initiated by adding 20 μl diluted xanthine oxidase. The plate was incubated at room temperature for 20 min, and the absorbance was read at 450 nm. The activity of SOD was calculated following the manufacturer’s instructions.

Serum amyloid P measurement

Mouse serum amyloid P (SAP) was assayed using an ELISA kit purchased from Immunology Consultants Laboratory, Newberg, OR. Serum and renal cortex lysates were diluted 1:1500, whereas urine was diluted 1:15 with reagent diluent. A total of 100 μl diluted samples or standards were added to anti-SAP precoated plates and incubated for 1 h at 22°C. A total of 100 μl appropriately diluted enzyme conjugated secondary Ab was added to each well and incubated at 22°C for another hour. Tetramethylbenzidine was used as substrate, and the reaction was stopped by adding 100 μl stop solution. The color was quantified by determining the absorbance at 450 nm and converted to absolute units using a standard curve.

PG D synthase and PGD2 measurement

PG D synthase (PGDS) was detected using an mAb purchased from Cayman Chemicals (Ann Arbor, MI). B6.Sle1.Sle3 (26) and MRL.lpr mice with spontaneous lupus nephritis as well as experimental anti-GBM disease were studied. FACS analysis was initiated by adding 20 μl diluted radical detector, and the reaction was continued by adding 100 μl stop solution. The color was quantified by determining the absorbance at 450 nm and converted to absolute units using a standard curve.

Renin and pro tease activity measurement

Renin levels in the urine were assayed using western blot, using a commercially available Ab (Research Diagnostics, Concord, MA), as described above. Total protease activity was measured with an enzyme activity-based Protease Screening kit (G-Biosciences, Maryland Heights, MO), which uses a dye-labeled protein substrate. Any proteases present in the urine sample effectively digest the protein substrate and release dye-labeled peptides. The absorbance of dye-labeled peptide is measured at 570 nm for determination of total protease activity. Both urine and renal cortex lysates were examined using this assay, following manufacturer’s instructions.

Real-time RT-PCR

Four-month-old B6, B6.Sle1.Sle3, and MRL.lpr mice were sacrificed, and RNA was extracted from the renal cortex (after stripping it away from the medulla) for real-time RT-PCR assays, as described previously (23); PGDS: 5′-TTTGTCCCAATTGGCCATCAGG-3′ and 5′-TTTGAATGTGGGCTGACGTGTGC-3′; SAP: 5′-TTTGGTCTAAGTGAAGGCTTGGG-3′ and 5′-TGACCTGTAACACTCCCTCAGGTA-3′; SD-
Fold-change was calculated as described previously (23). Statistics

Student *t* tests or the nonparametric Mann-Whitney *U* tests were conducted to compare continuous variables between two groups, such as the groups with and without anti-GBM disease. Multiple linear regression analyses were done to examine the association between glomerular pathology and the levels of the different molecules such as PGDS, renin, total protease, SAP, and SOD. Pearson correlation coefficients were computed to investigate the correlations among GN scores, renal activity index, and the levels of protease, PGD2, SOD, and SAP. ANOVA tests were conducted to compare the levels of PGD2, SAP, and SOD among the three strains of mice (B6, MRL.lpr, and B6.Sle1.Sle3 mice). Bonferroni corrections were used for multiple comparisons. Stepwise linear regression and stepwise ordinal logistic regression analyses were conducted to identify significant independent markers for disease activities such as GN score and renal activity index.

Results

**Upregulated excretion of various proteins during experimental immune nephritis**

We initiated the urine proteome-based biomarker studies with an experimental model of immune-mediated nephritis, anti-GBM nephritis, because disease sets in with reproducible kinetics in this model (23). Thus, 14 d following challenge with anti-GBM Abs (with LPS), B6 mice exhibit severe proteinuria, azotemia, and GN (23). To track the evolution of the urinary proteome over the course of the disease, two-dimensional gel electrophoresis was performed using 24-h urine samples obtained on days 0, 3, 7, and 14 following anti-GBM experimental nephritis induction. As shown in Fig. 1, there was no significant difference between the day 0 and day 3 urinary proteomes. However, a substantial number of proteins appeared in the urine from day 7 onwards, with peak levels being attained 14 d following disease induction, by which time point >100 protein spots were readily discerned in the urine.

To study these spots further, a region of interest was first demarcated (isoelectric point [pI] = 5.2–6.9, m.w. range = 18–116 kDa), encompassing the vast majority of the displayed spots but excluding the protein streaks corresponding to the major serum protein, albumin (Fig. 1, top panel). Within the selected region of interest, there were a total of 126 discrete protein spots that were visible to the ImageMaster software (GE Healthcare) used to quantify these spots. All 126 spots were excised for mass spectrometry-based identification, as exemplified for four of the proteins in Fig. 2. Based on their peptide sequence identities, we deduced that these 126 protein spots had originated from a total of 71 different proteins, as detailed in Table I.

As listed in Table I, these 71 proteins included various proteases (ubiquitin specific peptidase 1, kallikrein, renin, endopeptidases, dipeptidyl peptidase, trypsinogen, and aspartate protease), anti-proteases (serine proteinase inhibitor and cysteine-rich protease inhibitor), oxidative stress-related proteins (thyroid peroxidase, Cu/Zn SOD, ceruloplasmin), enzymes (glycine decarboxylase, carboxylesterase precursor, flavin-monooxygenase 1, esterase 1, amylase-α, PG-H2 D-isomerase, PG D2 synthetase, acyl-CoA dehydrogenase precursor), serum proteins (albumin, transferrin, Igs), acute phase reactants (SAP, macroglobulin, haptoglobin, α-fetoprotein), and shed receptors (e.g., integrins). Also elevated...
were several members of the renin-angiotensin system (renin, angiotensinogen, angiotatin), and TGF-β–related molecules (Smad1, Smad4, and TGF-β–induced proteins).

Validation studies—urinary PGDS, renin, SAP, and SOD are elevated in experimental anti-GBM nephritis

Ideally, it would be optimal to validate using orthogonal assays the urinary elevations observed in all 71 mass spectrometry-identified proteins. As this would not be practical, four molecules (PGDS, renin or total protease, SAP, and SOD) were selected for validation, based on the following criteria:

a. Abs or reagents are commercially available for assaying these molecules;

b. All of these molecules have documented roles in inflammatory diseases including nephritis and could potentially be produced within the kidneys, extrapolating from literature reports;

c. The selected molecules are representative of different biochemical pathways; hence, this selection may allow us to potentially screen multiple pathogenic events occurring during nephritis; and

d. Most of these molecules are of relatively low m.w.; hence, the appearance of these molecules in the urine is unlikely to be simply reflective of leakage due to a compromised glomerular filtration barrier.

Consistent with the findings on two-dimensional gels, PGDS was noted to be elevated in the urine, as determined by an orthogonal assay, Western blot (Fig. 3A). We also indirectly monitored PGDS activity by assaying the levels of its enzymatic product, PGD2. As can be seen in Fig. 3B, peak levels of urinary PGD2 were noted on day 14 and day 21 postnephritis induction, with the levels correlating well with glomerular pathology (Fig. 3C). To ascertain if the PGDS/PGD2 was originating from the kidneys or from the sera, corresponding serum levels were also examined. Serum PGD2 levels peaked on day 14 and returned to near basal levels by day 21 (Fig. 3D). Hence, at least on day 21, the kidneys rather than the serum appeared to be the major source of the urinary PGD2 (Fig. 3B versus 3D). This conclusion was fortified by the observation that renal cortical tissue collected on day 21 following anti-GBM disease also exhibited higher levels of PGD2 (Fig. 3E).

Using an orthogonal assay, Western blot, the increase in urinary renin during experimental immune nephritis was also validated (Fig. 4A). Because renin and a couple of additional proteases were noted to be elevated on the proteomic screen (Table I), we next assayed total protease activity in the urine using a colorimetric assay. Total urinary protease reached peak levels 14–21 d after induction of anti-GBM nephritis and correlated well with renal pathology (Fig. 4B, 4C). In contrast, the serum levels of protease were no higher than the basal serum levels at all time points during disease (Fig. 4D). Comparing the urinary and serum profiles of total protease, the major source of urinary protease in immune nephritis appeared to be renal in origin. Indeed, the renal cortex expressed increased levels of total protease following anti-GBM disease induction, as shown in Fig. 4E. Interestingly, in these anti-GBM–injected mice, total 24-h urine protease levels correlated fairly well with urine PGD2 ($r = 0.48$) and SOD ($r = 0.52$), but not with urine SAP ($r = 0.14$).
An orthogonal colorimetric assay was also used to confirm that urinary SOD was elevated during anti-GBM disease; this increase correlated well with renal pathology (Fig. 5A, 5B). However, serum SOD levels progressively fell over the course of the disease (Fig. 5C). Comparing the corresponding serum and urinary levels of SOD suggested that urinary SOD excreted later in disease may be largely renal in origin (Fig. 5B, 5C). Consistent with this, the renal levels of SOD also increased during disease (Fig. 5D). Finally, urine SAP was also validated to be increased, particularly on day 14 and day 21 of anti-GBM disease, using an orthogonal ELISA assay (Fig. 6). As for PGD2 and protease, urine SAP levels also correlated well with the GN score (Fig. 6). Whereas the serum may have been the dominant source of urinary SAP early in disease, urinary SAP on days 14–21 postdisease also appeared to be largely of renal origin based on the corresponding serum profiles of SAP and its elevated renal expression (Fig. 6).

Urinary PGD2, protease, SAP, and SOD are also elevated in spontaneous lupus nephritis

Having confirmed their elevation in experimental Ab-induced GN, we next assayed the urinary levels of PGD2, protease, SAP, and SOD in mice with spontaneous lupus nephritis. Six-month-old MRL.lpr and B6.Sle1.Sle3 mice develop renal disease following spontaneously arising lupus (24, 25). Compared to a healthy control strain, both mouse models of lupus nephritis exhibited elevated levels of all four molecules in their urine and the diseased kidneys (Fig. 7A–D). The levels of these four molecules were also examined in the sera of mice with lupus nephritis to address the possibility that some of the urinary molecules may be serum-derived in part. In the case of PGD2 and SAP, both molecules were also elevated in the serum of lupus nephritis mice (Fig. 7E, 7G), raising the possibility that the urinai contents of these two molecules may be serum-derived, at least in part. In contrast, total protease was not elevated in the serum of lupus mice, suggesting that the protease observed in the urine of mice with lupus nephritis may be predominantly, if not exclusively, of renal origin. SAP also exhibited different patterns of origint depending on the strain background; whereas urinary SOD appears to be predominantly renal in origin in B6.Sle1.Sle3 mice, it may be partly serum-derived in MRL.lpr mice (Fig. 7G, 7H). It is important to note that the elevation of all four molecules within the nephritic kidneys of lupus mice was also noted at the RNA level (Fig. 8). Finally, in contrast to the strains with lupus nephritis, the urinary levels of these four markers were barely elevated in the monocogenic strains with minimal disease, including B6.Sle1 and B6.Sle3 (Fig. 9).

In the above study, all mice were examined at 6 mo of age after disease onset. To test the predictive potential of these urinary molecules, we next examined B6.Sle1.Sle3 lupus prone mice at different ages, from 3 mo to 8 mo. These mice progressively became more nephritic, with the average GN scores evolving from 1 at 3 mo age to 2 at 4 mo, 2.5 at 5 mo, 3 at 6 mo, 3.5 at 7 mo, and 4 at 8 mo of age (data not plotted). Likewise, their respective renal pathology activity indices also progressively deteriorated from 0 to 2 at 3 mo age, 0 to 4 at 4 mo, 2.5 to 6.5 at 5 mo, 3.5 to 10.5 at 6 mo, 4.5 to 12 at 7 mo, and 7 to 10 at 8 mo age (data not plotted). We next examined how accurately 24-h proteinuria, blood urea nitrogen (BUN), and the urinary levels of PGD2, protease, SAP, and SOD reflected ongoing renal disease (as determined by histopathology) in this longitudinal series of mice/kidneys with progressively worsening nephritis. Overall, the urinary levels of all four molecules tested increased with age and disease in these longitudinal studies, correlating well with each other. For example, total 24-h urinary protease in these mice correlated well with urine PGD2 ($r = 0.67$), SOD ($r = 0.52$), and SAP ($r = 0.71$).
When the nephritis-predictive ability of the different urinary markers was compared with that of 24-h proteinuria and BUN, the markers clearly emerged superior. Whereas 24-h proteinuria and BUN correlated relatively poorly with the GN score ($r = 0.23$ and $0.19$, respectively), urine PGD2 ($r = 0.17$), total urine protease ($r = 0.42$), urine SOD ($r = 0.59$), and urine SAP ($r = 0.52$) correlated better with GN scores (Fig. 10). At the different ages examined, B6.Sle1.Sle3 mice did not exhibit a wide spread of renal pathology chronicity scores (range = 0–4; mean 1.2, SD 1.1); however, they exhibited a progressive worsening of renal pathology activity scores (range = 0–10.5; mean 5.7; SD 3.3). Urinary protease emerged as the strongest correlate of renal pathology disease activity ($r = 0.78$), with close seconds being PGD2 ($r = 0.62$) and SOD ($r = 0.65$) (Fig. 10). In contrast, 24-h proteinuria ($r = 0.04$) and BUN ($r = 0.28$) correlated poorly with the renal pathology activity index (Fig. 10). Adopting an alternative algorithm, we next examined which individual marker or combination of markers offered the highest disease predictive potential using FIGURE 3. Urine PGDS/PGD2 is elevated in anti-GBM disease. PGDS/PGD2 levels in urine (A and B), serum (D), and kidneys (E) of anti-GBM–challenged B6 mice are displayed. PGDS was assayed by Western blot in A, and PGD2 was assayed using colorimetric assays in B–E. Correlation between urine PGD2 and the corresponding GN score in all anti-GBM–challenged mice is plotted in C. Each dot represents data from an individual mouse. Horizontal bars denote group means. Day 0 (i.e., no anti-GBM challenge) data were compared with postchallenge values, using the Student t test. *$p < 0.05$; **$p < 0.001$.

FIGURE 4. Urine renin/protease is elevated in anti-GBM disease. Renin/protease levels in urine (A, B), serum (D), and kidneys (E) of anti-GBM–challenged B6 mice are displayed. Renin was assayed by Western blot in A, and protease was assayed using colorimetric assays in B–E. Correlation between urine protease and the corresponding GN score in all anti-GBM–challenged mice is plotted in C. Each dot represents data from an individual mouse. Horizontal bars denote group means. Day 0 (i.e., no anti-GBM challenge) data were compared with postchallenge values using the Student t test. **$p < 0.01$; ***$p < 0.001$. 2188 SOD, SAP, PGDS/PGD2, AND PROTEASE IN LUPUS NEPHRITIS by guest on November 8, 2017 http://www.jimmunol.org/ Downloaded from http://www.jimmunol.org/
stepwise linear regression analysis. Urine SAP \((p < 0.009)\) emerged as the single most predictive marker of histological GN, whereas urine protease \((p < 0.0006)\) and age in months \((p < 0.001)\) emerged as the two independent parameters that best predicted worsening renal pathology activity scores (data not plotted).

**Discussion**

Given the observation that early treatment of nephritis in SLE can significantly improve disease outcome \((6–8)\), early detection of renal involvement in lupus is of paramount importance. Because mediators of renal disease may be expected to be excreted in the urine, the urine potentially constitutes a window into the pathogenic events ongoing during nephritis. Indeed, other urinary molecules such as VCAM-1, MCP-1, and C3d have been suggested to be potential biomarkers of renal disease in SLE \((19, 26–28)\). The present comprehensive and unbiased screen for urinary biomarkers of nephritis in SLE has uncovered PGDS/PGD2, protease, SAP, and SOD as four potential biomarkers of disease, both in experimental anti-GBM disease as well as spontaneous lupus nephritis, with interesting differences among these molecules in terms of their biology and their predictive potential.

PGD2 is an acidic lipid mediator derived from arachidonic acid by sequential action of cyclooxygenase and PGD2 synthase. Hemopoietic PGD2 synthase is present in mast cells, Th2 cells, and other leukocytes, and it is thought to be responsible for the bulk of PGD2 production during allergic responses \((29–32)\). PGD2 is rapidly metabolized to PGJ2 and PGF2α, which retain significant chemoattractant properties, particularly for Th2 cells \((33)\). PGD2 has been implicated in the initiation and progression of inflammation. The injection of PGD2 into skin has been shown to result in erythema, edema, induration, and leukocyte infiltration \((34)\). PGD2 and other vasodilator PGs may also contribute to inflammation by increasing local blood flow. Fujitani et al. \((35)\) reported that lipocalin-type PGDS transgenic mice exhibited enhanced allergic airway inflammation. Ogawa et al. \((36)\) reported that in a diabetic rat model, urinary excretions of lipocalin-type PGDS increased preceding diabetic nephropathy and could predict the progression of renal injury, with similar findings being noted by others \((37)\). Because lipocalin-type PGDS has a smaller m.w. than serum albumin (i.e., it can readily filter through the GBM barrier), it may be expected to appear in the urine even before albuminuria.

**FIGURE 5.** Urine SOD is elevated in anti-GBM disease. SOD levels in urine (A), serum (C), and kidneys (D) of anti-GBM-challenged B6 mice are displayed. SOD was assayed using colorimetric assays. Correlation between urine SOD and the corresponding GN score in all anti-GBM-challenged mice is plotted in B. Each dot represents data from an individual mouse. Horizontal bars denote group means. Day 0 (i.e., no anti-GBM challenge) data were compared with postchallenge values using the Student t test. *\(p < 0.05\); ***\(p < 0.001\).

**FIGURE 6.** Urine SAP is elevated in anti-GBM disease. SAP levels in urine (A), serum (C), and kidneys (D) of anti-GBM-challenged B6 mice are displayed. SAP was assayed using ELISA. Correlation between urine SAP and the corresponding GN score in all anti-GBM-challenged mice is plotted in B. Each dot represents data from an individual mouse. Horizontal bars denote group means. Day 0 (i.e., no anti-GBM challenge) data were compared with postchallenge values using the Student t test. *\(p < 0.01\); ***\(p < 0.001\).
In our studies of experimental immune nephritis and spontaneous lupus nephritis, we found increased levels of PGD2 in the urine, serum, and renal cortex, and this increase correlated well with kidney disease. Although initial urinary PGD2 in the experimental anti-GBM disease model appeared to be serum-derived, the kidneys appeared to be a more dominant contributor later in disease. Our studies of spontaneous lupus nephritis also suggest that urinary PGD2 may be derived in part from the serum (Fig. 7). Finally, in the completed longitudinal studies in spontaneous murine lupus nephritis, the ability of urine PGD2 to predict renal pathology was quite impressive, although it was inferior to the predictive potential of some of the other markers tested in this study. Extrapolating from the published literature (as discussed above), we hypothesize that the increased PGDS/PGD2 in lupus nephritis may have a pathogenic role in renal inflammation.

A functional role for the renin-angiotensin system has also been implicated in the context of lupus nephritis. The inhibition of this axis using an angiotensin-converting-enzyme inhibitor, captopril, has been shown to be effective in improving survival, glomerular damage, proteinuria, lymphoid hyperplasia, dermatitis, and hypergammaglobulinemia in two mouse models of spontaneous lupus, MRL.\textsubscript{lpr} and NZW/NZB (38–40), with parallel findings in human lupus nephritis (41, 42). Increased renal renin activity has also been reported in patients with lupus nephritis (43, 44).

![Figure 7](http://www.jimmunol.org/)

**FIGURE 7.** Urine PGD2, protease, SAP, and SOD are elevated in urine (A–D), serum (E–H), and total kidneys (I–L) in two different strains with spontaneous lupus nephritis, MRL.\textsubscript{lpr} and B6.\textsubscript{Sle1.Sle3}, examined at the age of 6 mo (\(n=6–22\) each). Each dot represents data from an individual mouse. Horizontal bars denote group means. B6 values were compared with the values derived from the lupus mice using the Student \(t\) test. \(*p < 0.05\); \(**p < 0.01\); \(***p < 0.001."

![Figure 8](http://www.jimmunol.org/)

**FIGURE 8.** PGDS, SAP, SOD, and renin are expressed within nephritic kidneys. RNA from the renal cortex of B6, B6.\textsubscript{Sle1.Sle3} and MRL.\textsubscript{lpr} mice (\(n=5\) each) was isolated and assayed for message levels of the four molecules by real-time PCR. Fold change is reported with respect to the message levels in B6 and was calculated after normalization against GAPDH message, as detailed elsewhere (23). Indicated \(p\) values pertain to Student \(t\) test (or Mann-Whitney \(U\) non-parametric test where the data were not normally distributed) comparing the values in lupus mice against the B6 values. \(*p < 0.05\); \(**p < 0.01\); \(***p < 0.001."
studies are consistent with the above reports, indicating that elevated renal and urinary renin may be cardinal features of experimental anti-GBM disease and spontaneous lupus nephritis. Renin and related aspartyl proteases were found to constitute \( \sim 16.3\% \) of the total protease in our two-dimensional gel experiment, as surmised from densitometric scans (data not shown). In addition to renin (and aspartyl proteases), other proteases uncovered by the two-dimensional gel screen include dipeptidyl peptidase, kallikreins, and trypsinogen, some of which have been implicated in end-organ disease (44, 45). Of relevance, Dellalibera-Joviliano et al. (45) investigated the kallikrien levels in 30 patients with active lupus nephritis and 30 healthy controls and found that plasma and urine kallikrein activity was significantly higher in patients with active lupus nephritis compared with controls. In addition, another class of protease that has been implicated in the pathogenesis of lupus nephritis is matrix metalloproteinase (46, 47).

FIGURE 9. Urine PGD2, protease, SAP, and SOD are not elevated in monocongenic B6.Sle1 and B6.Sle3 mice. Twenty-four-hour urine samples from B6.Sle1 and B6.Sle3 mice at the age of 6 mo (n = 5) were collected, and the four molecules were assayed. Each dot represents data from an individual mouse. Horizontal bars denote group means. The dotted lines denote the group means in mice with lupus nephritis, as presented in the previous figures. B6 values were compared with the values derived from the lupus-congenic mice using the Student \( t \) test.

FIGURE 10. Disease-predictive potential of novel biomarker candidates in spontaneous murine lupus nephritis. Lupus-prone B6.Sle1.Sle3 mice were examined at the age of 3 mo (n = 3), 4 mo (n = 4), 5 mo (n = 3), 6 mo (n = 4), 7 mo (n = 4), and 8 mo (n = 3) for evidence of renal disease and urinary levels of various molecules. Displayed are the correlation profiles between GN score and proteinuria (A), renal disease activity index and proteinuria (B), GN score and BUN (C), and renal disease activity index and BUN (D). Also displayed are the correlations of urine PGD2, urine protease, urine SOD, and urine SAP with renal disease activity index (E–H) or with the corresponding GN score (I–L). Each dot represents a single mouse.
Given the wide spectrum of proteases implicated in nephritis, it is perhaps not a surprise that total protease activity was elevated in the kidney and urine of mice with experimental anti-GBM disease as well as spontaneous lupus nephritis. Another unique feature of urine protease that distinguishes this marker from all of the other markers examined in this study is the observation that it appears to be exclusively renal-derived in spontaneous lupus nephritis (Fig. 7B, 7F), and it exceeds the predictive potential of the other markers tested, as well as currently used markers, including proteinuria and BUN for predicting acute nephritis.

SAP and the closely related molecule, C-reactive protein (CRP), are highly conserved acute phase plasma proteins belonging to a group of proteins called pentaxins. SAP is a key acute phase protein in mice, whereas CRP assumes this role in humans (48). SAP has opsonizing properties and has been shown to bind DNA, chromatin, and nuclear debris released by necrosis and apoptosis and also to Clq and FcγR (49–52). Connolly et al. (53) have documented that plasma SAP is elevated in MRL/lpr, NZB/NZW, and NZB mice in relation to the onset and severity of lupus and subsequent loss of renal function. However, it has been shown that administering CRP to MPR/lpr mice markedly delayed the onset of proteinuria and lymphadenopathy, reduced renal pathology, increased survival, and reduced the levels of autoantibodies (54). SAP−/− mice spontaneously develop antinuclear autoimmunity with severe lupus-like GN (55), suggesting that SAP may have a protective role in lupus. Our findings are consistent with the published literature on SAP and indicate that the levels of SAP in the blood, urine, and kidneys closely parallel disease both in experimental anti-GBM disease and spontaneous lupus nephritis.

Extrapolating from the literature, it appears that the elevated SAP in the current study may not be pathogenic but may have a disease protective role in immune-mediated renal disease. Of interest, in a cross-sectional study of 610 patients with SLE, human serum CRP was noted to be associated with anti-DNA Abs, lupus anticoagulant, gastrointestinal manifestations, and anemia, suggesting that serum CRP might be a useful biomarker for identifying high-risk patients with SLE (56). Although our findings are consistent with these reports, an important caveat should be recognized. Because serum SAP is also elevated in anti-GBM disease (Fig. 6) and spontaneous lupus (Fig. 7), it is not clear if urine SAP levels might have the capacity to distinguish systemic versus renal inflammation in lupus.

Reactive oxygen species secreted by activated neutrophils and other cells can potentially cause injury to normal tissue (57). Under oxidative stress, SOD serves as a defense mechanism by degrading superoxides and attenuating local inflammation (57). SOD is one of many physiological antioxidants, serving as the first line of defense against oxygen-derived free radicals (58). Wang et al. (59) have documented that human lupus nephritis is associated with higher glomerular SAP, particularly in diffuse proliferative lupus nephritis. Likewise, Taysi et al. (60) demonstrated that the SOD activity was higher in serum of patients with SLE compared with healthy controls. Of note, the disease activity index correlated negatively with serum SOD in those patients, suggesting that SOD may be protective in human SLE (60). Our findings are consistent with the published literature on SOD and indicate that the levels of SOD in the blood, urine, and kidneys closely parallel disease, both in experimental anti-GBM disease and spontaneous lupus nephritis.

Extrapolating from the literature, it appears that the elevated SOD seen in these models may not be pathogenic but may have a disease protective role in immune-mediated nephritis. In this context, it would be important to test the therapeutic efficacy of SOD mimetics in lupus nephritis. Intriguingly, in spontaneous lupus, serum SOD is elevated in some strains (e.g., MRL/lpr) but not others (e.g., B6.Sle1.Sle3). In the latter strain, where urine SOD is likely to be predominantly renal in origin, it is noteworthy that urine SOD correlates well with GN score and renal disease activity indices (Fig. 10), performing better than 24-h proteinuria and BUN.

In summary, these unbiased two-dimensional gel-based proteomic studies have uncovered a handful of urinary molecules that have the potential to serve as biomarkers of immune-mediated nephritis, including lupus nephritis. Among these, urine protease emerges as the most promising marker, surpassing other candidate molecules examined in this report, as well as currently used laboratory yardsticks of lupus nephritis. Compared to the other markers examined in this report, PGD2/PGDS, SAP, and SOD, as well as a quartet of urinary markers reported in our earlier study (VCAM-1, P-selectin, soluble TNF receptor I, and CXCL16) (19), urine protease emerges as the only marker thus far examined that appears to be exclusively renal in origin. Clearly, longitudinal studies are warranted to firmly establish if the molecules uncovered in this study are superior to currently used yardsticks in predicting renal flares in SLE.

Disclosures

The authors have no financial conflicts of interest.

References


