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Elevated Urinary VCAM-1, P-Selectin, Soluble TNF Receptor-1, and CXC Chemokine Ligand 16 in Multiple Murine Lupus Strains and Human Lupus Nephritis

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In an effort to identify potential biomarkers in lupus nephritis, urine from mice with spontaneous lupus nephritis was screened for the presence of VCAM-1, P-selectin, TNFR-1, and CXCL16, four molecules that had previously been shown to be elevated in experimental immune nephritis, particularly at the peak of disease. Interestingly, all four molecules were elevated ~2- to 4-fold in the urine of several strains of mice with spontaneous lupus nephritis, including the MRL/lpr, NZM2410, and B6.Sle1/lpr strains, correlating well with proteinuria. VCAM-1, P-selectin, TNFR-1, and CXCL16 were enriched in the urine compared with the serum particularly in active disease, and were shown to be expressed within the diseased kidneys. Finally, all four molecules were also elevated in the urine of patients with lupus nephritis, correlating well with urine protein levels and systemic lupus erythematosus disease activity index scores. In particular, urinary VCAM-1 and CXCL16 showed superior specificity and sensitivity in distinguishing subjects with active renal disease from the other systemic lupus erythematosus patients. These studies uncover VCAM-1, P-selectin, TNFR-1, and CXCL16 as a quartet of molecules that may have potential diagnostic significance in lupus nephritis. Longitudinal studies are warranted to establish the clinical use of these potential biomarkers. The Journal of Immunology, 2007, 179: 7166–7175.

Systemic lupus erythematosus (SLE)3 is a systemic autoimmune disease where nephritis accounts for a substantial degree of the morbidity and mortality, both in patients and in mouse models of the disease (1–3). Unfortunately, the clinical management of nephritis in this autoimmune disease is still a difficult problem (3). One thing that is certain is that early diagnosis (and treatment) of lupus nephritis is associated with better disease outcome (4). Hence, it would be very beneficial if one could detect the presence of nephritis early in disease. Although serial renal biopsies may be ideal in closely monitoring the progression of renal disease (5), this may not be the most practical or feasible approach. Clearly, there is an urgent need for a “surrogate” marker of renal disease that one could potentially use to predict the onset of immune nephritis and to monitor its progression in lupus, as emphasized by others (6).

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 urine 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 urine protein-creatinine ratios appear to correlate well with each other (~95% correlation), and represent a well-accepted marker of renal disease that is currently available for clinical use (7). Supplanted with readouts from urinalysis (cells/casts), serum creatinine levels, and kidney biopsy information, the physician is able to plan an appropriate management strategy for the patient.

Emerging biomarkers with potential diagnostic value in lupus that have recently been reported include serum levels of various cytokines, mediators, or adhesion molecules (8–10), gene expression profiles in urine cells (11–13), and urine levels of chemokines (14, 15) and VCAM-1 (16). The present study was initiated based on our recent observation that VCAM-1, P-selectin, soluble TNFR-1 (sTNFR-1), and CXCL16 were elevated in the urine during experimentally induced immune nephritis in mice, correlating well with disease. This experimental model, which is induced by the administration of rabbit anti-glomerular Abs (17), is strain dependent (18, 19). We recently noted that strains that developed more severe disease following the antiglomerular insult exhibited higher urinary levels of VCAM-1, P-selectin, sTNFR-1, and CXCL16 (20). Given that these molecules were previously observed to be generated at least in part in the kidneys and appeared to have pathogenic relevance, it is important to ascertain whether these molecules are also elevated in the urine during spontaneous lupus nephritis. To this end, we monitored the urinary levels of these four molecules in several different strains of lupus mice. Finally, because VCAM-1, P-selectin, sTNFR-1, and CXCL16 appeared to be good markers of renal disease in murine lupus, we proceeded to examine whether these molecules were also elevated in the urine in human lupus nephritis.
Materials and Methods

Mice

C57BL/6 (B6), MRL/lpr, BXSB, and NZM2410 mice were purchased from The Jackson Laboratory or Taconic Farms. B6.Sle1.lpr mice were bred in our mouse colony as described (21). Urine from mice with spontaneous lupus was collected at 6 mo of age, or at the indicated ages. Female mice were used for all experiments, and 24-h urine collections using metabolic cages were conducted for all mice. All animal experiments were conducted in accordance with institutional guidelines.

ELISA detection of sTNFR-1, CXCL16, P-Selectin, and VCAM-1

The following ELISA kits were purchased from R&D Systems and used as dictated by the manufacturer’s protocol (mouse sTNFR-1 DuoSet, catalog no. DY425; mouse CXCL16 DuoSet, catalog no. DY643; human sTNFR-1 DuoSet, catalog no. DY225; human CXCL16 DuoSet, catalog no. DY1164; human VCAM-1 DuoSet, catalog no. DY809, human P-Selectin immunoassay kit, catalog no. BBE6; human MCP-1, catalog no. DY279; human urokinase-type plasminogen activator (uPAR), catalog no. DY807; human IL-1, catalog no. DY201; human IL-6, catalog no. DR206). All urine samples were diluted 1/5 or higher for the ELISA, and the concentrations of the respective molecules were ascertained using manufacturer supplied standards. Serum samples were diluted 1/100 for the ELISA. Likewise, all culture supernatants were diluted 1/2 or 1/10 before assaying for the released mediators. The absolute concentrations were determined by multiplying the ELISA-determined values by the respective dilution factors.

Kidney sections obtained from 2- or 6-mo-old mice were stained with the following primary Abs: goat anti-mouse CXCL16 Ab (no. AF503; R&D Systems), goat anti-mouse TNFR-1 Ab (no. BAF425; R&D Systems), goat anti-mouse VCAM-1 (no. BAF643; R&D Systems) Ab, or goat anti-mouse P-selectin Ab (no. SC-6941; Santa Cruz Biotechnology) or the respective isotype control Abs, and developed as described previously (21). Briefly, Ag retrieval was performed using a sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20 (pH 6.0)) in a pressure cooker, protein-blocked for 20 min, and peroxidase-blocked for 20 min. For primary incubation, anti-goat P-selectin Ab (0.2 μg/ml, 60 min), anti-P-selectin Ab (0.2 μg/ml, 60 min), anti-CXCL16 Ab (0.25 μg/ml, 60 min), or anti-VCAM-1 Ab (0.3 μg/ml, 30 min) were added. This was followed by incubation with biotinylated anti-goat IgG 2 μg/ml, ABCelte staining (Vectastain ABCelte kit, HRP), diaminobenzidine chromagen, and hematoxylin. The protein block, diaminobenzidine chromagen, and peroxidase block were purchased from DakoCytomation (catalog no. K4011).

Patient samples

Urine and sera from healthy adults (n = 15, average age = 43), SLE patients (n = 38; with or without active nephritis; average age = 34), and rheumatoid arthritis (RA) patients (n = 6, average age = 61) were drawn at the Albert Einstein College of Medicine, in accordance with institutional review board approved guidelines. All patients and controls (except for one) were females. Most of the individuals in all groups were of Hispanic or African-American origin. Disease activity was gauged using SLE disease activity index (SLEDAI) scores (22). The patients exhibited a wide spread of SLEDAI scores displayed in Table I. The degree of lupus nephritis was determined by standard clinical (serology, renal function, urinary sediment, and protein) and/or pathological (kidney biopsy) criteria. All patient studies were conducted with informed consent and institutionally approved guidelines.

Renal SLEDAI

Kidney disease activity was assessed using the renal SLEDAI score that consists of the four kidney-related criteria of the SLEDAI (22) (i.e., hematuria, pyuria, proteinuria, and urinary casts). The presence of each one of these four parameters yields a score of 1–4 points, depending on the severity; thus, the renal SLEDAI score can range from 0 (in nonactive renal disease) to a maximal score of 16.
Statistics

Groups were compared against each other using the Student t test where the data were normally distributed. Otherwise, the nonparametric Mann-Whitney U test was used. Most statistical tests including correlation coefficients (R) were calculated using Excel or GraphPad Prism. The nonparametric receiver operating characteristic (ROC) curves were calculated using Intercooled Stata version 9.2 (StataCorp). The area under the ROC curves ("AUC") were reflective of the specificity and sensitivity of the marker, with values of 0.7–0.8, 0.8–0.9, and 0.9–1.0 representing “acceptable,” “excellent,” and “outstanding” discriminatory potential, respectively.

Results

VCAM-1, P-selectin, sTNFR-1, and CXCL16 are elevated in the urine in spontaneous lupus nephritis

B6.Sle1.lpr, MRL.lpr, and NZM2410 mice develop high levels of anti-dsDNA and anti-glomerular autoantibodies, accompanied by severe lupus nephritis, and early mortality (21, 23–25). When the above quartet of molecules was assayed, it was evident that urine from these strains of mice suffering from spontaneous lupus nephritis also harbored increased levels of these molecules (Fig. 1A). Indeed, the 24-h urine levels of these molecules were reflective of the extent of renal disease in these mice (Fig. 1B). Specifically, the urine levels of protein correlated well with the corresponding urine levels of VCAM-1 (R = 0.8, p < 0.001), CXCL16 (R = 0.78, p < 0.001), and P-selectin (R = 0.75, p < 0.001), but less so with TNFR-1 levels (R = 0.57, p < 0.001) (Fig. 1B).

Urinary VCAM-1, P-selectin, sTNFR-1, and CXCL16 are higher than the corresponding serum levels

Given the observation that VCAM-1, P-selectin, sTNFR-1, and CXCL16 were elevated in the urine of lupus mice, we next examined the degree to which these molecules may be serum-derived, focusing initially on the MRL.lpr strain. It was clear that with disease, the serum levels of all four molecules increased 2- to 4-fold, relative to predisease 2-mo-old MRL.lpr mice and B6 controls (Fig. 2). It is remarkable that the serum levels of VCAM-1 and P-selectin in 6-mo-old MRL.lpr mice ranged from 1 to 5 mg/ml and 0.25–0.75 mg/ml, respectively, levels that were significantly higher than the levels of the other two molecules. These findings raise the possibilities that the urinary content of these molecules may in part be serum derived.

To ascertain whether any of these molecules were further enriched in the urine relative to the serum, the urine-serum ratios of these molecules were next calculated, after normalizing both values against albumin. The degree of enrichment of the four molecules in urine followed this order: VCAM-1 > P-selectin > CXCL16 > TNFR-1 (Fig. 2). Whereas VCAM-1 was enriched 2- to 10-fold in the urine relative to the serum in 6-mo-old MRL.lpr mice, TNFR-1 was hardly so. P-selectin and CXCL16 exhibited
intermediate degrees of enrichment in the urine, suggesting that they maybe partly serum derived and partly kidney derived. Similar urinary enrichment of these molecules were also noted in two additional strains which develop spontaneous lupus, B6.Sle1.lpr and BXSB, in an age- and disease-dependent fashion, as illustrated for VCAM-1 and CXCL16 (Fig. 3).

VCAM-1, TNFR-1, P-selectin, and CXCL16 are hyperexpressed within the diseased kidneys in lupus

Given that most of the above molecules were enriched in the urine during the course of lupus, we wondered whether they may be originating from the kidneys. To ascertain this, we next examined the levels of these molecules in renal cortical lysates from MRL.lpr and BXSB mice with spontaneous lupus nephritis. As depicted in Fig. 4, all four molecules were expressed in the renal cortical lysates from both these strains with spontaneous lupus nephritis. Immunohistochemistry studies further highlighted the fine localization of these four molecules within the renal cortex (Fig. 5). Diseased MRL.lpr kidneys exhibited VCAM-1 staining in the peritubular capillaries, vascular bundles, endothelium, and scattered glomerular cells as well as in interstitial inflammatory cells. Staining of the same cells at a weaker level was noted in 2-mo-old MRL.lpr kidneys before disease onset (Fig. 5 and data not shown). The remaining three molecules, P-selectin, sTNFR-1, and CXCL16, showed a fairly similar distribution, being observed in the distal tubules, collecting ducts, endothelium, the interstitial inflammatory cells, and scattered glomerular cells. Once again, staining was more prominent in diseased MRL.lpr kidneys, compared with 2-mo-old MRL.lpr or B6 control kidneys (Fig. 5).

VCAM-1, TNFR-1, P-selectin, and CXCL16 are also elevated in the urine of SLE patients

To determine whether this quartet of molecules was elevated in the urine of SLE patients, urine samples from 38 SLE patients with a spread of SLEDAI scores (Table I), 6 RA patients, and 15 normal healthy controls were studied for the expression of these four molecules, using commercially available ELISA kits. As shown in Fig. 6A, urine from patients with SLE showed significantly higher levels of urinary VCAM-1, CXCL16, TNFR-1, and P-selectin, compared with urine from RA patients and normal controls. Importantly, urine levels of these molecules showed excellent correlation with concurrent urinary protein-creatinine ratios (Fig. 6B) and SLEDAI scores (Fig. 6C). Not surprisingly, the urinary levels of all four molecules exhibited excellent ROC profiles, with AUC values (ranging from 0.76 – 0.89) reflecting high degrees of sensitivity and specificity for distinguishing SLE patients from healthy controls (Fig. 7).

Because patients with elevated SLEDAI may or may not necessarily have renal disease, we next focused on SLE patients with active nephritis. Importantly, SLE patients with active nephritis exhibited the highest levels of these molecules in the urine, particularly VCAM-1 and CXCL16. Thus, patients with increased SLEDAI and active renal disease (SLEDAI >0; n = 18) had significantly higher levels of urinary VCAM-1/creatinine (618 vs 370 pg/mM, p < 0.02) and CXCL16/creatinine...
(154 vs 85 pg/mL, \( p < 0.04 \)), compared with patients with elevated SLEDAI but without active nephritis (SLEDAI \( \geq 6 \), renal SLEDAI \( = 0 \); \( n = 5 \); Fig. 8). Because both these groups of patients (with nephritis vs those without) had SLEDAI scores that were not statistically different (average of 12.5 and 10.4, respectively), the differences in the urinary levels of these molecules were not simply due to differences in SLEDAI scores. Examined using an alternative approach, urinary VCAM-1, sTNFR-1, and CXCL16 exhibited “outstanding” sensitivity and specificity AUC values (0.91–0.93) for discriminating SLE patients with active renal disease, as is evident from the ROC curves of these molecules (Fig. 9 and data not plotted).

We next examined the levels of VCAM-1, CXCL16, TNFR-1, and P-selectin in the sera of these patients after distinguishing those with active disease from the rest. Several important observations were made. First, the levels of all molecules were elevated in the sera of SLE patients with active but not inactive disease (Fig. 10). Second, it was clear that VCAM-1, CXCL16, TNFR-1, and P-selectin were further enriched in the urine, relative to the serum, only among the lupus patients with active disease (Fig. 10), closely mirroring the findings in lupus mice.

Finally, we compared the performance of the above markers to that of four additional molecules, IL-1, IL-6, uPAR, and MCP-1. The first three represent inflammatory mediators that have previously been studied in several end-organ diseases, whereas MCP-1 is a chemokine that has previously been documented to be a potential biomarker in lupus nephritis (15). IL-1 and IL-6 were barely detected in the urine samples studied (data not shown); in contrast, significant levels of uPAR were detected in these samples. However, as shown in Fig. 11A, urinary uPAR levels were not significantly different in the different SLE patient groups (e.g., nephritis vs non-nephritis, or active vs
inactive disease), and were barely different from the corresponding levels in healthy controls. In contrast, urinary MCP-1 was higher in the urine of SLE patients compared with normal urine (Fig. 11B). However, these levels were not significantly higher in the urine of nephritic SLE patients (Fig. 11B) compared with the urinary levels in inactive or non-renal SLE. The corresponding ROC AUC values for urinary MCP-1 and uPAR for distinguishing active renal lupus from nonrenal lupus were 0.78 and 0.77, respectively (data not plotted).

FIGURE 6. Urine VCAM-1, P-selectin, sTNFR-1, and CXCL16 are also elevated in human lupus nephritis. A, Spot urine samples from 38 SLE patients (Table I), 15 healthy adults, and 6 RA patients were assayed for the levels of VCAM-1, P-selectin, sTNFR-1, and CXCL16. Indicated p values pertain to Student’s t test or Mann-Whitney U test comparisons of the urinary levels of each molecule in patients against that in normal controls (indicated below the x-axis), or SLE vs RA, as indicated at the top (*, p < 0.05; **, p < 0.01; ***, p < 0.001). Shown also are the correlation profiles of these urinary molecules (normalized to urine creatinine) against the corresponding urine protein-creatinine ratios (B) and SLEDAI scores (C). R: correlation coefficient.

FIGURE 7. Specificity/sensitivity ROC curves pertaining to the use of urinary VCAM-1 (A), CXCL16 (B), sTNFR-1 (C), and P-selectin (D) for discriminating SLE patients from normal controls. The data shown in Fig. 6 were used to generate the shown ROC curves, which plot the specificity and sensitivity profiles of the indicated urinary molecules in distinguishing SLE patients from normal controls. Indicated are the corresponding AUC values.
Discussion

Given that early intervention in lupus nephritis is associated with better outcome, it is imperative that we diagnose the disease activity in the kidney as early as possible. Currently, urinary albumin levels, supplanted by additional laboratory tests, constitute the cornerstone of diagnostic screening in lupus nephritis. There have been isolated reports that the urinary levels of certain chemokines, notably MCP-1, or adhesion molecules, notably VCAM-1, might have the potential to serve as additional biomarkers for lupus nephritis (8, 14–16). The present study supplements this list with additional excreted markers, P-selectin, sTNFR-1, and CXCL16, which are reproducibly excreted in the urine in spontaneous murine and human lupus nephritis. Given that these molecules may also be expressed within the diseased kidneys, these molecules may also be playing critical roles in disease pathogenesis.

VCAM-1 is an adhesion molecule that is expressed on a large number of cell types including macrophages, dendritic cells (DCs), endothelial cells, etc., and plays an important role in cell recruitment into tissues mediated by VCAM-1:VLA4 interactions. Increased expression of VCAM-1 has also been noted in arthritis and other immune disorders where it has also been identified as a therapeutic target (26, 27). It has previously been observed to be expressed in the kidneys, both in murine and human lupus (28–30), and this is reinforced by our present study that shows VCAM-1 staining in murine lupus kidneys. Our findings resonate well with a previous report of increased urinary VCAM-1 in lupus nephritis (16). Though serum levels of VCAM-1 increased significantly with disease both in murine and human lupus, the kidney appeared to be an additional source of VCAM-1, based on the immunohistochemistry and renal expression studies and the enrichment in the urine particularly in active disease. The present findings raise hope that VCAM-1 may be a reliable urinary marker of active disease both in mice and patients with nephritis, thus confirming earlier reports.

P-selectin is an adhesion molecule that is expressed on platelets and other cell types, and has been reported to be elevated in other autoinflammatory diseases, where it has also been recognized as a circulating disease marker (31–33). It has been observed to be expressed in lupus kidneys, both in mice and in patients (34, 35), but it has not been specifically measured in lupus urine. Hence, this represents the first report of elevated urinary P-selectin in lupus nephritis. Though serum levels of P-selectin increased significantly with disease both in murine and human lupus, the kidney appeared to be an additional source of P-selectin based on the immunohistochemistry and renal expression studies and the enrichment in the urine, in the murine studies.

TNFR-1 is a cytokine receptor that plays various functions in the immune system. Importantly, increased serum TNFR-1 has been noted to be increased in human SLE (36, 37). There is a single report of its being hyperexpressed in the kidneys in proliferative lupus nephritis (38), but it has not been examined in the
urine as yet. Hence, this represents the first report of elevated urinary sTNFR-1 in lupus nephritis. However, urinary TNFR-1 in murine and human lupus nephritis may be originating from the serum at least in part, based on our findings, consistent with our findings in the experimental anti-glomerular basement membrane (GBM) model (20).

CXCL16 is a chemokine, reported to be expressed on DCs and macrophages, that is important for the recruitment of T cells and NK T cells into various tissues, and for cell:cell interactions via the CXCR6 counterreceptor. It also appears to be important in arthritis (39–41); however, it has not been studied before in lupus, either in the kidneys or in the urine. Hence, this represents the first report of its increased presence in lupus urine. Though serum levels of CXCL16 increased significantly with disease both in murine and human lupus, urinary CXCL16 may be at least partly generated in the kidneys based on the renal expression studies. The immuno-histochemistry studies revealed that the above quartet of molecules are expressed by multiple cell types in the kidneys, including endothelial cells, tubular cells, glomerular cells as well as infiltrating leukocytes.

We believe the inflamed kidney represents an important source of these molecules in the urine, based on the following observations. First, these molecules are enriched in the urine, relative to the blood, both in murine and human lupus (Figs. 3 and 10). Second, these molecules are expressed within the renal parenchyma, based on findings from two orthogonal studies (Figs. 4 and 5). Third, patients with active renal lupus had the highest urinary levels of these molecules (Figs. 6 and 8). Finally, these molecules have high specificity and sensitivity in discriminating active renal lupus from nonrenal disease, as indicated by their high ROC AUC values (Fig. 9). In addition, it appears likely that the above molecules may be serum derived in part, arising as a consequence of systemic inflammation. To begin with, these molecules are elevated in the serum, both in mice and patients with SLE. Additionally, RA patients exhibit urinary (and serum) levels of these molecules that are significantly higher than the levels in normal mice (Fig. 6). These observations suggest that systemic inflammation may result in increased levels of circulating VCAM-1, P-selectin, sTNFR-1, and CXCL16.

Given that levels of these molecules correlate well with proteinuria or disease severity both in mice and patients, it is imperative that we assess the diagnostic use of assaying these molecules in the urine. Even if the serum may constitute a significant source of these molecules in the urine, the ability to detect these molecules in the urine could have a tremendous impact on clinical diagnostics. Not only is urine a far more convenient body fluid to procure, in some clinical settings it may be the only fluid available (e.g., in field hospitals or from pediatric patients). However, it is clear that serum is not the only source of urinary VCAM-1, P-selectin, sTNFR-1, and CXCL16.

Given the diagnostic potential of these molecules, it is imperative to systematically monitor the urinary levels of these molecules...
were divided into four groups: 1) inactive SLE (SLEDAI ≤ 5), renal SLEDAI = 0; 2) mild-active, nonrenal SLE (SLEDAI = 1–5, renal SLEDAI = 0); 3) severe-active, nonrenal SLE (SLEDAI ≥ 6, renal SLEDAI = 0); and patients with severe lupus nephritis, an observation that begs the question of whether these molecules are actually mediating the pathogenesis of nephritis. Third, we have recently demonstrated that the coordinate blocking of one or more of these molecules may offer therapeutic relief that is even more superior in lupus nephritis. Based on our Ab blocking studies in the experimental anti-GBM nephritis model, CXCL16 may turn out to be yet another therapeutic target in nephritis (20). Finally, it remains to be seen whether the coordinate blocking of one or more of these molecules may offer therapeutic relief that is even more superior in lupus nephritis.

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Disclosures
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References


