Decreased renal expression of vascular endothelial growth factor in lupus nephritis is associated with worse prognosis

Yingyos Avihingsanon[1,2], Thitima Benjachat[1], Adis Tassanarong[3], Pimpayao Sodsai[1], Vipawee Kittikovit[1,4] and Nattiya Hirankarn[1,5]

[1]Renal Division, Lupus Research Unit, Department of Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand; [2]Department of Medicine, Chulalongkorn University, Bangkok, Thailand; [3]Department of Medicine, Faculty of Medicine, Thammasart University, Pathumthani, Thailand; [4]Department of Pathology, Chulalongkorn University, Bangkok, Thailand and [5]Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

Recent studies suggest that vascular endothelial growth factor (VEGF) plays a crucial role in the preservation of renal function and may also serve as a useful biomarker in monitoring the progression of lupus nephritis (LN). Here we sought to correlate intrarenal VEGF expression with renal histopathology and prognosis of LN. Biopsy specimens from 35 patients with Class III or IV LN (ISN/RPS categorization) were found to have lower levels of intrarenal VEGF than those found in biopsy tissue taken from 10 donor kidneys sampled at the time of allograft reperfusion. This reduced amount of VEGF mRNA in the patients with LN negatively correlated with glomerular endocapillary proliferation, crescent formation, and a high histologic activity index but was positively associated with increased numbers of urinary podocytes. The level of intrarenal VEGF mRNA accurately predicted the deterioration of renal function in these patients within 12 months. Our study shows that expression of VEGF in renal tissue may serve as a molecular marker of renal damage and may be a predictive factor for short-term loss of kidney function in patients with LN.

Correspondence: *Yingyos Avihingsanon, Renal Division, Lupus Research Unit, Department of Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand. E-mail: yingyos.a@gmail.com*

Proliferative lupus nephritis (LN) is the most common and severe histology of LN.[1] Steroids and cytotoxic drugs remain the most commonly used treatments despite the many associated adverse events.[2] Renal histology is essential for the selection of proper treatment and prognostication of the disease.[3] Specific histological findings, such as crescent formation, may determine prognosis.[3] However, immunosuppressive therapy could mitigate such pathology and therefore improve renal prognosis.[4] Since the discovery of the molecular mechanisms of systemic lupus erythematosus (SLE), novel immunosuppressive therapies have been introduced.[5] Nevertheless, the mechanism of the loss of renal function remains unknown.[6] Studies of intrarenal molecular signatures could reveal the molecular mechanism of the disease and predict renal prognosis.[7–9] Finally, molecular classification may be integrated into the histological classification of LN.

The integrity of the glomerular and peritubular capillaries is vital for renal function. Progressive capillary loss, with obliteration of the microvasculature, frequently accompanies fibrosis, which is a characteristic feature of progressive renal disease.[10,11] Progression of glomerulopathy is, at least in part, due to loss of glomerular integrity. Vascular endothelial growth factor (VEGF) promotes survival, proliferation, and differentiation of glomerular endothelial cells.[12] Decreased expression of VEGF has been associated with various glomerulopathies such as crescentic glomerulonephritis, focal glomerulosclerosis, IgA nephropathy, pre-eclampsia, and aging kidneys.[10,13,14] Administration of VEGF has been shown to stabilize kidney function in many models including the remnant model, thrombotic microangiopathy, and chronic cyclosporine nephropathy.[15–18] The protective actions were principally mediated through preservation of glomerular and peritubular capillary structures.[18] A recent study in human diabetic nephropathy has confirmed the role of VEGF in maintaining renal vasculature and identified it as a novel biomarker.[19] The growing evidence supports the potential role of VEGF in SLE and nephritis, but the studies remain inconclusive.[13,20–23]

Intrarenal quantitative gene expression may be used for grading of disease severity.[8] For instance, intrarenal transforming growth factor-β (TGF-β) gene expression could determine progression of chronic kidney disease.[9] In LN, it is difficult to determine prognosis at the time of renal flare. In an earlier study, we suggested a serial non-invasive measurement of urinary mRNA for chemokines and growth factors that could predict the prognosis of this disease.[24] In this study, we aim to determine an association between intrarenal molecular signatures and renal histology. Furthermore, we determined whether the molecular signature in the kidney could predict the progression of LN.

RESULTS

Patients

Fifty-one biopsy samples were obtained during a diagnostic process of clinically active LN. Ten samples were excluded due to an inadequate number of glomeruli (less than five) or chronic scarring glomeruli. Six samples from patients with LN class V excluded. Thirty-four samples were from female patients. Mean (s.e.) age was 31 (1.27) years. The mean (s.e.) SLE disease activity index (SLEDAI)[25] and level of serum creatinine, 24 h urine protein, and erythrocyturia were 10.58 (0.91), 1.74 (0.26) mg per 100 ml, 3.53 (0.47) g/day, and 32.70 (15.47) cells per high power field, respectively (Table 1).

Ten samples of implantation biopsies from donor kidneys were used as controls. Six kidney samples were from living

donors, and four were from deceased donors (Table 1). Histological examination of the wedge-biopsies showed unremarkable findings except for minimal tubular injury.

Renal histology of LN

Eight samples were class III and 27 were class IV by the ISN/RPS classification of LN, respectively. More details of renal histology are shown in Table 1. The mean (s.e.) of renal activity and chronicity indices were 7.49 (0.85) and 4.06 (0.53), respectively. Crescent formation was observed in 12 samples (34%). Other pathologies observed in the samples were endocapillary proliferation (82%), fibrinoid necrosis (34%), glomerular neutrophil infiltration (65%), and thrombotic microangiopathy (16%).

Intrarenal expression of VEGF in LN

The levels of VEGF mRNA in the kidneys of LN patients ($n = 35$) were decreased as compared with the implantation biopsies of kidney donors ($n = 10$) (-0.64 ± 0.05 vs -0.08 ± 0.13 log copies; $P < 0.001$) (Table 1). The levels of heme-oxygenase-1 (HO-1) were decreased in LN (-0.45 ± 0.06 vs 0.30 ± 0.26 log copies; $P = 0.002$), whereas the levels of TGF-β and angiopoeitin-1 (ANGPT-1) were not different between patients and controls (-0.13 ± 0.06 vs -0.03 ± 0.15 log copies; $P = 0.22$ and (0.60 ± 0.08 vs 0.16 ± 0.38 log copies; $P = 0.44$).

Intrarenal VEGF mRNA levels were lower in samples with crescent formation (-0.98 ± 0.03 vs -0.62 ± 0.05 log copies;

Table 1 | Clinical and histological variables at the time of biopsy[a]

	Controls[b]	Patients	*P*-value
Number	10	35	
Gender (female/male)	2/8	34/1	
Age (years)	33.40 ± 3.76	31.74 ± 1.27	0.74
Clinical parameters			
Serum creatinine (mg per 100 ml)	1.18 ± 0.13	1.74 ± 0.26	0.93
Proteinuria (g/day)	0	3.53 ± 0.47	<0.001
Urinary erythrocyte count (per high power)	0	32.70 ± 15.47	<0.001
MDRD-GFR (ml/min per 1.73 m²)[c]	81.10 ± 9.80	66.36 ± 6.84	0.49
SLEDAI[d]	NA	10.58 ± 0.91	
Steroid dose (mg/day)	0	32.20 ± 6.64	<0.001
Activity index	NA	7.49 ± 0.85	
Chronicity index	NA	4.06 ± 0.53	
Renal histology			
III (S), (G)	NA	7, 1	
IV (S), (G)	NA	10, 17	
III (A), (A/C)	NA	4, 4	
IV (A), (A/C)	NA	16, 11	
Intrarenal mRNA levels			
VEGF	−0.08 ± 0.13	−0.64 ± 0.05	<0.001
HO-1	0.30 ± 0.26	−0.45 ± 0.06	0.002
TGF-β	−0.03 ± 0.15	−0.13 ± 0.06	0.22
Angiopoeitin-1	0.16 ± 0.38	0.60 ± 0.08	0.44

[a]Data are expressed as mean ± s.e.
[b]Control: implantation biopsy from six living donors and four deceased donors.
[c]MDRD-GFR, glomerular filtration rate at the time of biopsy.
[d]SLEDAI, Systemic Lupus Erythematosus Disease Activity Index.[25]
A, active; C, chronic; G, global; HO-1, heme-oxygenase-1; NA, not applicable; S, segmental; TGF-β, transforming growth factor-β; VGEF, vascular endothelial growth factor.

$P = 0.04$). The biopsy samples with diffuse endocapillary proliferation ($\geqslant 25\%$ of glomeruli) expressed lower VEGF levels (-0.72 ± 0.05 vs -0.44 ± 0.06 log copies; $P = 0.003$). Samples with a high activity score (score $\geqslant 3$ of total 24) expressed lower VEGF levels (-0.70 ± 0.05 vs -0.40 ± 0.07 log copies; $P = 0.009$). However, samples with glomerular neutrophil infiltration ($\geqslant 25\%$ of glomeruli) showed no difference in VEGF levels (-0.67 ± 0.08 vs -0.62 ± 0.05 log copies; $P = 0.09$). Figure 1a–d shows that the presence of crescent formation, endocapillary proliferation, and a high activity index were associated with decreased VEGF mRNA levels.

Correlation between VEGF, TGF-β, AGPT-1, and HO-1
As there is a functional relationship among genes in the hypoxia-inducible pathway, such as VEGF, TGF-β, AGPT-1, and HO-1, we attempted to determine the association between each mRNA level in the biopsy tissues. Figure 2a–c shows that mRNA levels of VEGF were associated with TGF-β ($R = 0.41$, $P = 0.02$) and HO-1 ($R = 0.65$, $P < 0.0001$), but not ANGPT-1 ($R = 0.15$, $P = 0.41$) mRNA levels.

Immunohistochemistry localization of VEGF within renal biopsies
In kidney donors, the VEGF protein was expressed on podocytes and markedly expressed on tubular epithelial cells (Figure 3c and e). In LN, weak VEGF staining was observed in all samples (Figure 3b, d and f). The VEGF protein was scarcely seen on glomeruli and tubular epithelial cells of samples with diffuse endocapillary proliferation (Figure 3b) or crescent formation (Figure 3d).

Intrarenal VEGF mRNA levels predict a loss of renal function within 12 months
Active LN patients were treated with standard therapy (see Materials and Methods) and were followed for 12 months after kidney biopsy. Of all 35 patients, 10 patients experienced a loss of their renal function within 12 months (doubling serum creatinine levels or end-stage renal disease (ESRD)). We performed receiver operating characteristic analysis to determine the best cutoff that had the maximal sensitivity and specificity based on a loss of renal function. The mRNA cutoff level of -0.63 log copies could predict a loss of renal function with negative and positive predictive values of 100 and 53%, respectively. We observed that 53% of patients with low VEGF mRNA levels, but none of the patients with high levels, experienced loss of their renal function within 12 months. Patients with low VEGF mRNA levels had a significantly increased risk of loss of renal function, including doubling serum creatinine levels, ESRD, or both events combined ($P < 0.001$ by log-rank test) (Figure 4a–c). In contrast, crescent formation and a high renal activity index were not associated with ESRD (data not shown).
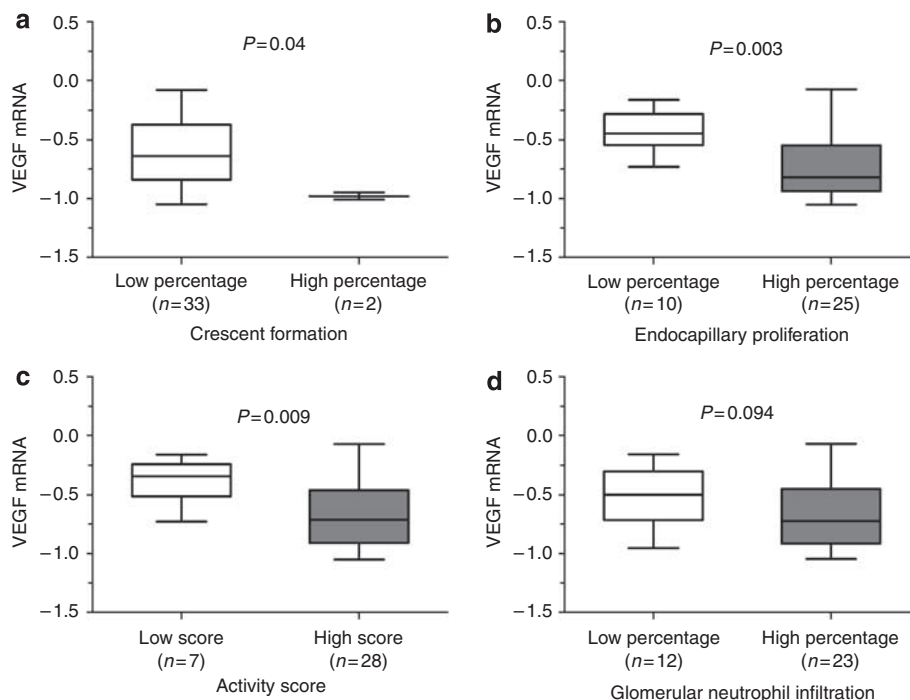

Figure 1 | Intrarenal vascular endothelial growth factor (VEGF) mRNA and renal pathology. Box and whisker plots show the 10th, 25th, 50th (median), 75th, and 90th percentiles of values (log) for VEGF mRNA levels in the kidney tissue of patients with class III/IV lupus nephritis. The levels of VEGF mRNA were significantly lower in samples with the presence of crescentic formation (**a**), endocapillary proliferation (> 25% of glomeruli) (**b**), and high activity index (AI > 3) (**c**), but not glomerular neutrophil infiltration (> 25% of glomeruli) (**d**). (*P*-value by Mann–Whitney test.) Numbers in the parentheses indicate the number of biopsy samples.
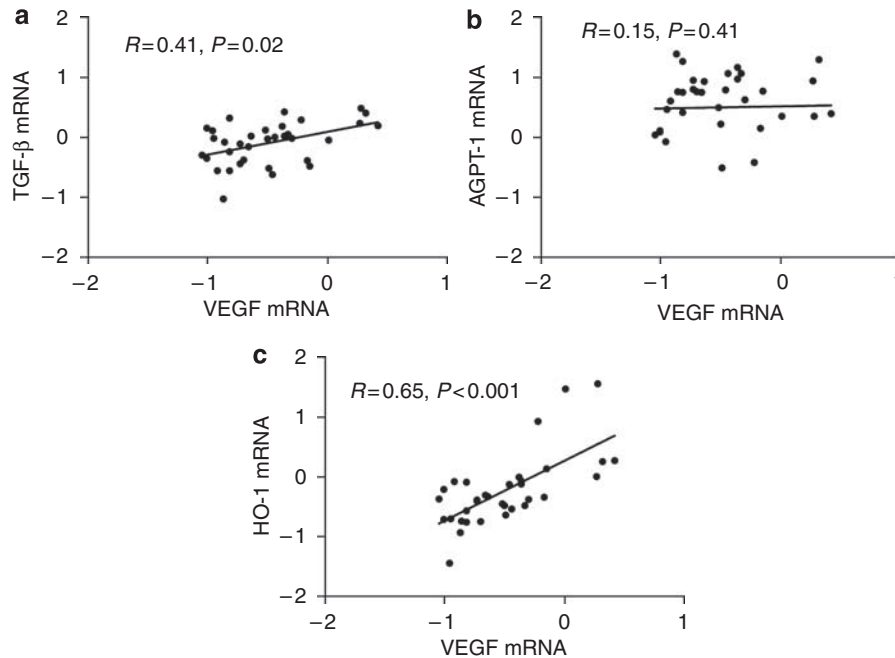
Figure 2 | Coordinated expression of intrarenal VEGF and related genes. (**a**) The relationship between the levels of VEGF and TGF-β was significant at $P = 0.02$ ($R = 0.41$). (**b**) The relationship between the levels of VEGF and ANGPT-1 was not significant ($P = 0.41$, $R = 0.15$). (**c**) The relationship between the levels of VEGF and HO-1 was significant at $P < 0.0001$ ($R = 0.65$).

Urinary loss of podocytes is associated with active LN

In an earlier study, we reported an increase in urinary VEGF levels in patients with biopsy-proven proliferative LN.[24] Although tubular epithelial cells are the main source of VEGF in the kidney, we hypothesized that the reduction of intrarenal VEGF in this study may partly be explained by a loss of podocyte cells into the urine. We therefore analyzed the relationship between mRNA levels from urine cells and renal tissue during active LN. To perform a proper comparison, urine samples were collected from patients ($n = 21$) on the day of renal biopsy. There was a significant association between urinary WT-1 (podocyte marker) and VEGF mRNA levels (Figure 5a) ($R = 0.51$; $P = 0.02$). Urine WT-1 and VEGF mRNA levels were increased in active LN (urine WT-1 = 2.88 ± 0.25 and VEGF = 2.14 ± 0.23 log copies). In contrast, intrarenal WT-1 and VEGF mRNA levels were decreased in active LN (renal WT-1 = -0.56 ± 0.08 and VEGF = -0.73 ± 0.14 log copies) (Figure 5b).

DISCUSSION

Renal histology study is essential for guidance of patient management and for predicting prognosis of renal disease.[26] Patients with proliferative LN (ISN/RPS class III or IV) are inevitably destined for chronic or end-stage kidney disease.[27,28] The use of currently available immunosuppressive treatment has significantly improved renal prognosis.[29] However, physicians have been unable to readily predict individual responses until patients finish a 6-month course of immunosuppressive treatment.[28] In general, a loss of renal function within 6 months after induction of treatment could determine long-term prognosis.[3] In this retrospective study,

intrarenal VEGF expression was decreased in severe LN. Moreover, VEGF expression at the time of renal flare may be a useful predictor of poor renal function within 12 months. Molecular biomarkers may be useful in the diagnosis and prognosis of LN in the future.

Several studies have suggested that VEGF plays a key role in endothelial cell proliferation and capillary repair.[16,18,30] In the model of membranoproliferative GN, blockade of the $VEGF_{165}$ protein could lead to progressive renal damage.[31] In the remnant kidney model, VEGF is reduced in both glomeruli and tubular cells, which could be corrected by VEGF replacement.[17] VEGF could enhance endothelial cell repair as well as increase angiogenic response of peritubular capillaries.[32] It is known that VEGF can alter endothelial cell growth, integrity, and function and may eventually contribute to glomerulopathy.[12,18,30] It is expected for reduced endocapillary proliferation to be associated with decreased VEGF. This study could not determine such an association.

Vascular endothelial growth factor has been shown to stabilize kidney function in animal models of thrombotic microangiopathy[16] and chronic cyclosporine nephropathy.[17] The protective actions were principally mediated through preserved glomerular and peritubular capillary structures.[18] This may help to preserve glomerular filtration rate by maintaining glomerular capillary filtration surface area as well as preventing tubulointerstitial fibrosis.[18] This vascular protective action of VEGF could explain, in this study, an association between intrarenal VEGF and a loss of renal function in 12 months. Furthermore, histological evidence in this study showed expression of VEGF in both the glomeruli and the tubular cells of kidney donors, whereas there was a
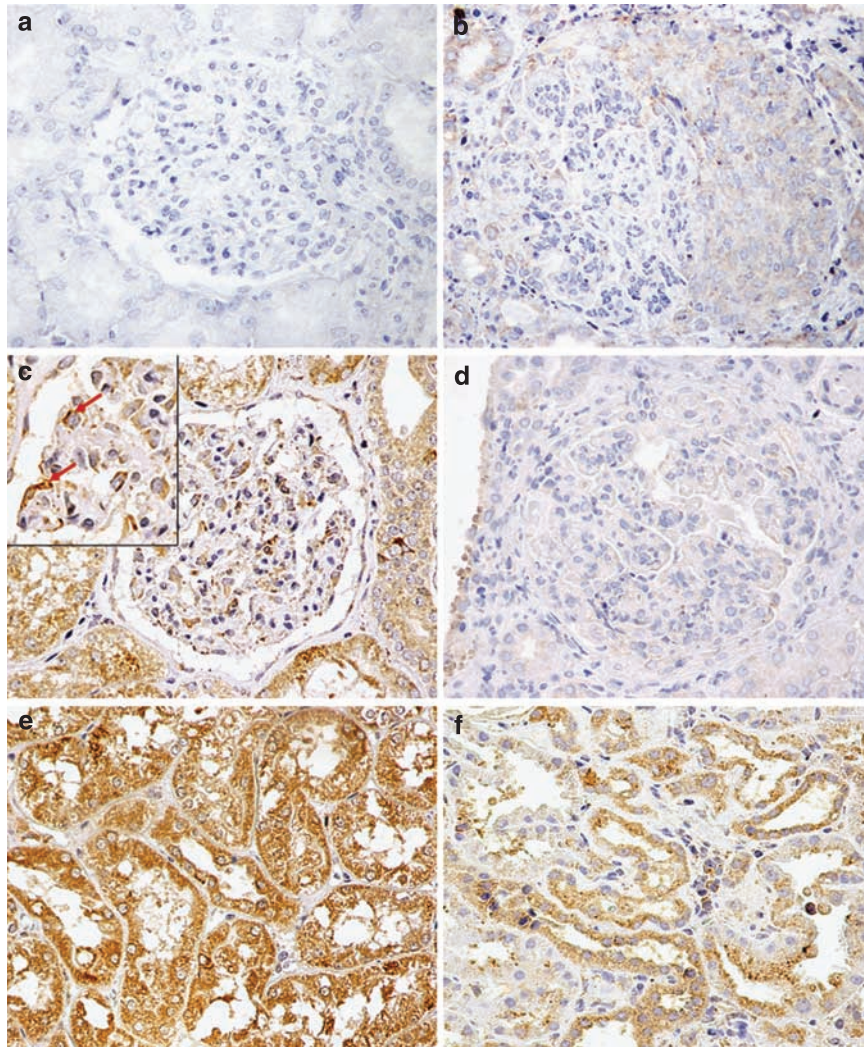
Figure 3│Immunohistochemistry localization of VEGF within renal biopsies of patients with lupus nephritis or kidney donors (implantation biopsy). (**a**) Sections were not stained with control monoclonal antibodies. (**b**) VEGF protein was absent in crescentic glomeruli of patients with lupus nephritis but markedly expressed in glomeruli of implantation biopsies of kidney donors (**c**); inset shows VEGF ± podocyte cells (arrows). (**d**) VEGF was scarcely seen in glomeruli with endocapillary proliferation of lupus nephritis. (**e**) VEGF was ubiquitously expressed on tubular epithelial cells of donor kidney tissues but was reduced in the tubulointerstitium of kidneys of patients with lupus nephritis (**f**). (Original magnification × 400, except inset × 600).

marked reduction of VEGF in both structures of kidneys with active LN.[13,33] A study of renal tubular cell lines found that VEGF acts as a survival factor by induction of cell proliferation and antiapoptotic responses.[34] The expression of VEGF in renal tubules may result to protect against injury such as hypoxia, ischemia/reperfusion, hypokalemia, or oxidative stress.[34–36]

In this study, intrarenal HO-1 expression was well correlated with VEGF expression. The mRNA levels of both genes were decreased in LN as compared with kidney donors. In human kidney transplantation, both HO-1 and VEGF mRNA levels have been shown to be decreased in deceased donors (prolonged ischemia time) as compared with living donors (short ischemia time).[33] In this study, the levels of VEGF decreased as the severity increased; therefore, further studies are needed to clarify the role of hypoxic injury in LN.

Studies of serum VEGF in SLE patients have shown higher levels of VEGF in patients with active SLE than in patients with inactive SLE or healthy individuals.[20–22] It is difficult to compare VEGF expression among the studies, as the quantification methods and studied samples (serum versus tissue) were different.[23] The local effects of VEGF may be different from the systemic responses. The variation of patients' characteristics and immunosuppressive treatments may be considered to be confounding factors. It should be noted that patients received a moderately high doses of steroids in this study, although we did not find a relationship between steroid dose and molecular profiling. Furthermore, serum VEGF levels may not be associated with intrarenal VEGF levels, as we may detect VEGF expression from different cell sources. For instance, the serum VEGF may originate from vascular endothelial cells, whereas renal VEGF,
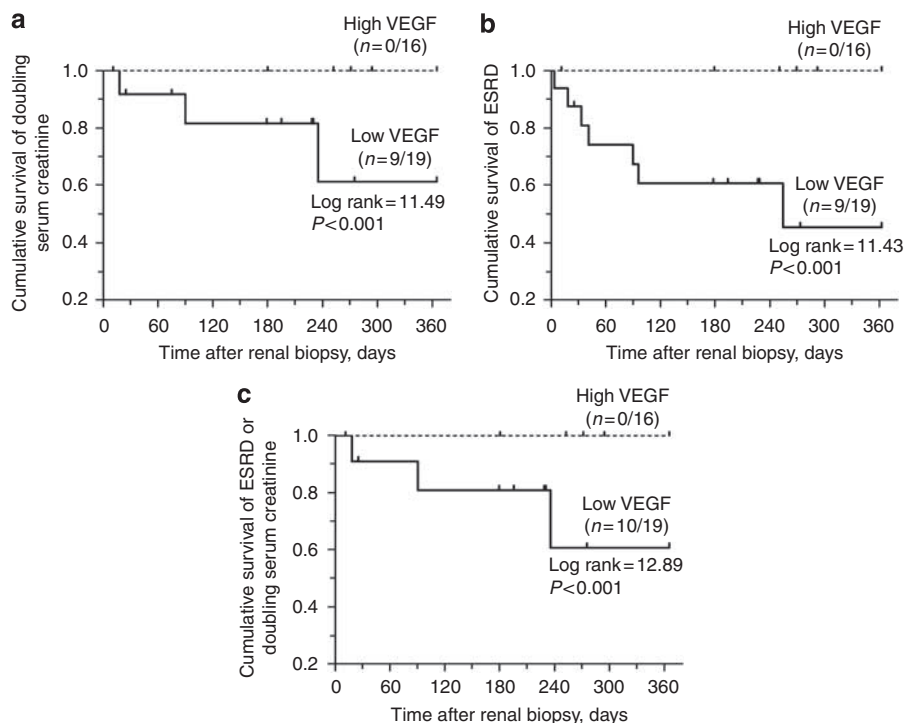
Figure 4 | Analysis of the relationship between VEGF mRNA levels and a loss of renal function in 12 months. (**a**) Shows Kaplan–Meier estimates of the time to doubling serum creatinine in all patients with class III/IV LN, as defined by two groups of VEGF mRNA levels (the best cutoff mRNA level identified by ROC analysis). (**b**) Shows Kaplan–Meier estimates of the time to end-stage renal disease (ESRD). (**c**) Shows Kaplan–Meier estimates of the time to both outcomes combined (doubling serum creatinine or ESRD). According to log-rank analysis, *P*-values were <0.001 for the model based on the two groups shown in all panels.
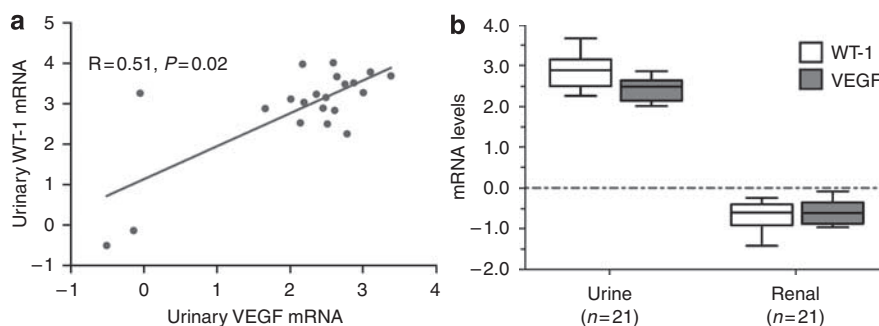

Figure 5 | Study of a podocyte marker (WT-1) and VEGF from urine samples of lupus nephritis patients. (**a**) The relationship between the levels of VEGF and WT-1 was significant at $P = 0.02$ ($R = 0.51$). (**b**) Box and whisker plots show the 10th, 25th, 50th (median), 75th, and 90th percentiles of values (log) for WT-1 and VEGF mRNA levels in the urine as compared with their levels in the kidney tissue of patients with class III/IV lupus nephritis ($n = 21$). The dotted line represents normal values.

in this study, originates from tubular epithelial cells and podocytes.[34,37] Finally, patients with different stages and levels of severity of SLE may show different patterns of VEGF expression.[13,22]

We previously reported an association between urinary VEGF expression and active proliferative LN,[24] whereas in this study, an inverse relationship between intrarenal VEGF and histological activity was found. This has been validated by performing mRNA studies from same-day collection of urine and renal tissue. Figure 5 shows an inverse relationship between urine and intrarenal VEGF mRNA levels. We hypothesize that there may be a significant urinary podocyte

loss in the active LN.[37] Although tubular epithelial cells are the main source of VEGF in the kidney, urinary podocyte loss may partly contribute to a reduction of VEGF in crescent formation.[37] Selective knockout of VEGF in the podocyte showed impaired glomerular capillary formation due to a loss of endothelial cells, supporting the important role of VEGF in podocytes in maintaining capillary integrity.[38] Yu *et al.*[39] showed that urinary podocyte loss is associated with glomerular damage in both primary and secondary glomerulonephritis in mice. We confirmed that there was urinary podocyte loss in LN by demonstrating an increase in urinary WT-1 mRNA levels of patients with active nephritis

(Figure 5). It would be interesting to further determine whether urinary podocyte numbers might be a functional biomarker for disease activity.

In conclusion, this study shows the pivotal role of renal VEGF expression in human LN. Patients with proliferative LN who had decreased intrarenal VEGF expression are at risk for a rapid decline of renal function. At the time of renal flare, the combination of renal pathology such as class III/IV LN and reduced VEGF expression could predict poor renal survival. Intrarenal VEGF may become a candidate surrogate marker for targeting therapy and in development of clinical trials.

MATERIALS AND METHODS
Patients
A total of 51 patients underwent renal biopsy for diagnostic evaluation of active LN between 2002 and 2005. All patients had been diagnosed with SLE according to the 1997 American College of Rheumatology criteria. All biopsies were examined by one pathologist (VK) who was not aware of the results of the molecular study. The samples were classified according to the histological types of LN using the ISN/RPS classification.[40] Six samples from patients with LN class V were excluded from the study. Ten samples were excluded because of inadequate number of glomeruli (less than five) or chronic scarring glomeruli. In class III or IV LN, patients were treated with oral prednisolone plus a 6-month course of intravenous cyclophosphamide[41] or oral mycophenolate mofetil.[42] In rapidly progressive renal failure from crescentic LN, three consecutive doses of intravenous methylprednisolone were given and three sessions of plasmapheresis were performed. The patients then received oral prednisolone plus a 6-month course of intravenous cyclophosphamide.[43]

The control group consisted of kidney samples from implantation biopsies after reperfusion of kidney allografts. Inclusion criteria were living or deceased donors with normal serum creatinine levels and donor age of less than 55 years. The kidney samples from patients with delayed graft function or prolonged ischemia time were excluded.

The study was approved by the Ethics Committee for Human Research of the Faculty of Medicine, Chulalongkorn University, and written informed consent was obtained from all patients.

Renal histology
Paraffin sections were stained with hematoxylin and eosin, periodic acid-Schiff, trichrome, and silver for light microscopy. The specimens were scored for activity and chronicity indices as described earlier.[1] The maximum scores of the activity index and chronicity index were 24 and 12, respectively. The activity index was the sum of semiquantitative scores of the following parameters: endocapillary proliferation, fibrinoid necrosis, cellular crescents, leukocyte infiltration, hyaline thrombi, and interstitial infiltration. The chronicity index was the sum of semiquantitative scores of the following parameters: glomerular sclerosis,

fibrous crescents, interstitial fibrosis, and tubular atrophy. A percentage of each parameter was calculated by the equation:

$$\text{Percentage of each pathology} = (\text{Number of involved glomeruli}/ \text{Total number of obtained glomeruli}) \times 100\%$$

RNA isolation from renal biopsy samples
Diagnostic renal biopsy specimens from LN patients were obtained and stored at $-80\,^{\circ}\text{C}$. RNA isolation, quantification, and reverse transcription into complementary DNA were performed as described earlier.[8]

Quantification of mRNA
The mRNA of 18s rRNA, VEGF, ANGPT-1, TGF-β, and HO-1 were measured using a Light Cycler machine (Roche Molecular Biochemicals, Indianapolis, IN, USA). The sequences of primers and fluorescence probes are as follows: 18s rRNA sense, 5′-gcccgaagcgtttactttga-3′; 18s rRNA antisense, 5′-tccattattcctagctgcggtatc-3′; 18s rRNA probe, 5′FAM-aaagcaggcccgagccgcc-TAMRA3′; VEGF sense, 5′-cctacagcacaa caaatgtgaatg-3′; VEGF antisense, 5′-caaatgctttctccgctctga-3′; VEGF probe, 5′FAM-caagacaagaaaatccctgtgggcct-TAMRA3′; ANGPT-1, sense 5′-tgcaaatgtgccctcatgtta-3′; ANGPT-1 antisense, 5′-tcccgcagtatagaacattcca-3′; TGF-β sense, 5′-ccctgcccc tacatttggag-3′; TGF-β antisense, 5′-ccgggttatgctggttgtaca-3′; TGF-β probe, 5′FAM-cacgcagtacagcaaggtcctggcc-TAMRA3′; HO-1 sense, 5′-gcccttcagcatcctcagttc-3′; HO-1 antisense, 5′-ggtttgagacagctgccacat-3′; HO-1 probe, 5′FAM-tgcagcaga gcctggaagacaccc-TAMRA3′. All primer pairs were designed to span across an intron–exon boundary to distinguish amplification of genomic DNA. Each PCR was carried out in a 20 μl reaction volume composed of 2 μl of cDNA template and 18 μl of a real-time PCR mastermix that contained 10 μl of 2 × QuantiTech Probe Mastermix (Qiagen Inc., Chatworth, CA, USA), 0.5 μM forward primer, 0.5 μM reverse primer, and 0.2 μM probe. No fluorescent signal was generated by these assays when genomic DNA was used as a substrate, which confirmed that the assays measured only mRNA. The levels of mRNA were analyzed by a comparative method.[44] The reference RNA was a pool of RNA from implantation kidney biopsies of live-donors. To control possible variation among PCR runs, VEGF and an 18s rRNA plasmid (housekeeping gene) were used as calibrators. The PCR amplicon for 18s rRNA was used for developing standard curves. The standard curves were based on the principle that a plot of the log of the initial target copy of a standard versus threshold cycles results in a straight line. The levels of mRNA were expressed as the number of copies per microgram of total RNA isolated from renal biopsy tissues.

Immunohistochemistry
For immunohistochemistry, tissue samples were fixed in 4% buffered paraformaldehyde and embedded in paraffin. Deparaffinized sections were heated in a microwave oven with sodium citrate buffer. The rabbit anti-VEGF-A antibody

sc152 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; dilution of 1:100) and an Envision reagent kit (Dako, Carpinteria, CA, USA) were used. The color product of peroxidase was developed by the 3,5 diaminobenzidine substrate and counterstaining with hematoxylin. Negative controls included normal rabbit IgG.

Quantitation of the urine podocyte marker and VEGF

To properly compare intrarenal and urinary gene expression, a 50 ml urine sample was collected on the day of kidney biopsy. The urine sample was immediately centrifuged after collection at 1000 g for 30 min at 4°C. Total RNA was isolated from the cell pellets using an RNA blood mini kit (Qiagen, Chatworth, CA, USA), measured for concentration, and reverse transcribed into complementary DNA as described earlier.[24,45] The mRNA levels of VEGF, WT-1 (podocyte marker), and 18s rRNA (housekeeping gene) were measured as described above.

Statistical analysis

Statistical analysis was performed using the SPSS software (version 11.5, SPSS Inc., Chicago, IL, USA). The levels of mRNA deviated significantly from the normal distribution ($P < 0.001$) and were reduced by log-transformation. All data are given as mean and s.e. The Mann–Whitney test was used for comparison between the two groups. The relationship between the mRNA levels of each group was estimated with Spearman's ρ correlation. A loss of renal function was determined by a doubling of serum creatinine calculated from the renal biopsy date or ESRD. The criteria of ESRD included having a calculated MDRD-GFR below 15 ml/min or initiation of renal replacement therapy (dialysis or transplantation). To distinguish patients who had a loss of renal function, a receiver operating characteristic curve of mRNA levels was used to determine the cutoff levels that maximized the combined sensitivity and specificity. We estimated the probabilities of a loss of renal function using the Kaplan–Meier method and compared them using a log-rank test. All *P*-values below 0.05 were considered statistically significant.

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