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Decreased urinary excretion of vascular endothelial growth factor in idiopathic membranous glomerulonephritis

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Background. Membranous glomerulonephritis (MGN) has, for unknown reasons, an unpredictable and highly variable clinical course. Vascular endothelial growth factor (VEGF) enhances endothelial cell proliferation, angiogenesis, microvascular permeability, and monocyte chemotaxis, and it activates proteinases. In normal kidneys, it is predominantly expressed by glomerular podocytes, where its physiological function and role in development of renal diseases are obscure. This study was designed to evaluate the urinary excretion of VEGF in MGN compared with several other glomerular disease and to asses its relationships to the clinical activity of MGN.

Methods. Urinary VEGF was studied during renal biopsy using a sandwich enzyme immunoassay from 30 patients with idiopathic MGN, 8 with minimal change glomerulonephritis, 10 with focal segmental glomerulosclerosis (FSGS), 8 with necrotizing glomerulonephritis associated with systemic vasculitis, and 12 with diabetic nephropathy. In addition, 33 healthy controls were examined. Fifteen patients with MGN were re-evaluated 12 months later, and the evolution of proteinuria was compared with changes in urinary VEGF excretion.

Results. In healthy control subjects, urinary VEGF excretion was 68 ± 10 (95% CI, 49 to 88) ng/mmol creatinine (U_{Cr}). In MGN, the excretion was decreased to 16 ± 3 (CI, 10 to 23) ng/mmol crea ($P < 0.0001$, ANOVA), whereas in minimal change glomerulonephritis and diabetic nephropathy, it was unchanged [55 ± 14 (CI, 24 to 86) and 101 ± 25 (CI, 45 to 156) ng/mmol U_{Cr}, respectively, P = NS]. In vasculitis and FSGS patients, the excretion was higher than normal [184 ± 68 (CI, 24 to 344), $P = 0.01$, and 160 ± 29 (CI 95 to 226), $P = 0.002$ ng/mmol U_{Cr}, respectively]. The excretion did not correlate with serum VEGF, renal function, or proteinuria. In the follow-up of 15 patients, improving MGN (decreasing proteinuria) was associated with increasing VEGF excretion, while persistent disease (no change or increase of proteinuria) was associated with constantly low urinary VEGF excretion. The change in urinary protein excretion over one year correlated inversely with the change in urinary VEGF ($r = -0.57$, $P = 0.026$).

Conclusions. MGN is associated with decreased urinary VEGF compared with normal subjects, which is in contrast with other proteinuric diseases. Moreover, decreasing clinical activity (proteinuria) is accompanied by increasing VEGF excretion. Urinary VEGF may serve as an indicator of activity of MGN.

Key words: growth factors, glomerulonephritis, proteinuria, immune process, sandwich enzyme immunoassay, vascular permeability.

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Membranous glomerulonephritis (MGN), a prototype of an immune-mediated glomerular disease, is characterized by abundant, nonselective proteinuria and highly individual course and prognosis [1]. The antigen(s) causing human MGN is unknown. However, in Heymann nephritis representing the experimental model of the disease, antibodies are directed against the megalin (gp330) receptor-associated protein complexes in the chlathrin-coated pits of the podocytes [2]. In the initial phases of the disease, the immune deposits are formed beneath the podocytic foot processes and are later incorporated into the glomerular basement membrane [3]. The immune process leads to generation of terminal complement complexes (C5a-9, membrane attack complex), which are essential for the development of proteinuria [4]. These complexes are capable of inducing the release of cytokines, oxidants, and proteinases, which may be responsible for the altered permeability of the glomerular capillary wall [5, 6].

Progression of MGN is linked to the duration and degree of proteinuria [7, 8]. On the other hand, proteinuria may resolve spontaneously [9] or as a result of immunomodulating therapy [10, 11]. Factors regulating protein excretion and the evolution of MGN are unknown.

Vascular endothelial growth factor (VEGF; vascular permeability factor) is a potent mitogen that enhances the proliferation of vascular endothelial cells, angiogenesis, and microvascular permeability [12, 13]. At least four VEGF isoforms have been found, and they interact with specific tyrosine kinase receptors *Flt-1, Flt-4*, and KDR/*Flk-1* on endothelial cells [14, 15]. In normal kidneys, VEGF is expressed in glomerular epithelial cells and weakly in distal tubular and connecting duct cells [16, 17], as well as in the juxtaglomerular areas [18]. Also, human mesangial cells and peripheral blood mono-

nuclear cells are capable of producing this molecule [19, 20]. Some recent studies have suggested that the expression of VEGF is abnormal in many glomerular and renal vascular diseases, as well as in renal allograft rejection [17, 21]. However, its role in renal pathophysiology is at present unclear.

To study the urinary excretion of VEGF in various glomerular diseases and normal subjects, we developed a novel sandwich enzyme immunoassay and correlated the excretion with several clinical parameters and plasma levels of VEGF. We also investigated the relationships between changes in clinical activity of MGN and urinary VEGF excretion.

METHODS

Study subjects

Patients. The study population comprised 30 patients with idiopathic MGN [22, 23]. There were 20 males and 10 females, and their mean age was 45 ± 2.9 (mean ± SE, median 43, range from 25 to 75) years. The protein excretion at the start of the study was 7.1 ± 0.7 (median 6.2, range from 2.2 to 15.6) g/24 h. The serum creatinine concentration was 91 ± 6.2 (median 81, range from 57 to 185) μmol/L, and the 24-hour creatinine clearance was 1.5 ± 0.1 (median 1.5, range from 0.7 to 2.4) mL/sec/1.73 m^2. Samples for the study were obtained at the time of morphologic diagnosis of MGN.

Fifteen patients with the nephrotic syndrome (proteinuria more than 3 g/24 h) were followed up for at least 12 months, and were studied both at diagnosis of MGN and one year later. Seven of the 15 patients had full (urinary protein excretion deceased to less than 0.5 g/24 h) or partial (protein excretion decreased by more than 50% compared with the initial values) remission during the follow-up. Three of the patients received immunomodulating treatment (pulse methyl prednisolone or cyclosporine). In eight patients, the abundant proteinuria either persisted or increased during the follow-up. Two of them were treated with immunomodulating drugs.

Controls. Twenty-four–hour urinary samples were obtained in 38 patients before the renal biopsy (where the diagnosis was done). Eight had minimal change glomerulonephritis [male to female ratio 3:5, mean age 48 ± 5.6 years, serum creatinine (S_{Cr}) 76 ± 5 μmol/L]. Ten had focal glomerulosclerosis (male to female ratio 5:6, mean age 39 ± 4.3 years, S_{Cr} 141 ± 39 μmol/L). Eight had necrotizing glomerulonephritis (male to female ratio 4:4, mean age 59 ± 4.7 years, S_{Cr} 308 ± 78 μmol/L) usually associated with systemic vasculitis, and 12 had overt (macroproteinuric) diabetic nephropathy (male to female ratio 10:2, mean age 54 ± 3.6 years, S_{Cr} 173 ± 16 μmol/L). In addition, the samples were obtained in 33 normal subjects (male to female ratio 21:12, mean age 37 ± 1.7 years) who were studied as outpatients.

Laboratory analyses

Urinary and serum samples. Urinary examinations were based of 24-hour collections throughout the study. These were obtained at the hospital prior to renal biopsy and (in 15 patients with MGN) 12 months later during outpatient visits. Aliquots of the 24-hour urine collection were centrifuged at 3000 r.p.m. for 10 minutes, and the clear supernatants were stored (without protease inhibitors) at −70°C. Venous blood samples were collected in prechilled tubes and centrifuged within 30 minutes, and serum samples were stored at −20°C.

VEGF assay. Urinary VEGF was determined using a sandwich enzyme immunoassay in which polyclonal goat antibodies to human VEGF (hVEGF; R&D Systems, Minneapolis, MN, USA) were precoated (0.5 μg/well) onto microtiter wells. Standards (recombinant hVEGF, 5000 to 0 ng/L; Genzyme Diagnostics, Cambridge, MA, USA), and samples were introduced to the wells. The VEGF in standards and/or samples, first bound to the coated antibody, was then detected by polyclonal rabbit antibody to hVEGF (Genzyme Diagnostics), followed by alkaline phosphatase-conjugated goat antibody to rabbit IgG (Boerhinger Mannheim Corp., Indianapolis, IN, USA). Unreacted components were removed by washings. A chromogen solution added to the wells formed a colored end product that was proportional to the amount of VEGF present in the samples. Serum VEGF was determined as mentioned previously in this article by using tenfold diluted sera. Detection limit of the assay was 10 ng/L, and for statistical purposes, values below this were assigned as 10 ng/L.

Precision of the VEGF assay. Intra-assay and interassay variations were 4.7 and 5.1% for the VEGF at a concentration of 1.5 ng/L, respectively, and 6.3 and 6.7% for the VEGF at the concentration of 0.3 ng/L, respectively.

Analytical recovery of VEGF. Analytical recoveries of added recombinant VEGF 0.1, 0.5, and 2.0 ng/L (initial VEGF content was 0.27 ± 0.01 ng/L) to aliquots (10 each) of pooled urine ranged from 80 to 100%. When added to urines from three patients with MGN, the analytical recoveries of 0.5 ng/L VEGF ranged from 86 to 123%.

Storage stability of VEGF. The pH values of urine were adjusted to 5.3, 7.0, and 9.0. VEGF was added to a final concentration of 0.5 ng/L. Aliquots of each sample were stored at −20°C, 4°C, and room temperature, and VEGF was analyzed after 1, 6, 13, and 20 days. There was no significant loss of VEGF immunoreactivity at pHs 5.3 and 7.0 during the storage at any temperature even for 20 days. At a pH of 9.0, there were 20 and 30% loss of immunoreactivity, respectively, during 6 and 20 days at −4°C. At −25°C, the loss of immunoreactivity was about 2% at pH 9.0 during 6 to 20 days of storage.

To compensate for alterations caused by varying uri-

nary concentration, the excretion of VEGF was related to concomitant urinary creatinine (U_{Cr}).

Other laboratory analyses. Urinary tumor necrosis factor-α (TNF-α) was analyzed using a solid-phase, double-antibody radioimmunoassay [24], and urinary transforming growth factor-β1 (TGF-β1) by an enzyme immunoassay [25]. Immunoturbidimetry was used to measure the concentrations of urinary albumin [26] and α_1-microglobulin [27]. Renal function was assessed by serum creatinine (normal values from 50 to 130 μmol/L) and (in MGN patients) by 24-hour creatinine clearance (normal values > 1.4 mL/sec/1.73 m^2). Serum creatinine, albumin, as well as the urinary total protein, albumin, and creatinine were measured using routine methods.

Statistical analysis

Values are expressed as mean ± SEM. Also, median values as well as 95% CI are shown where appropriate. One-way analysis of variance (ANOVA) was employed for repeated measurements, and comparisons between the two groups were performed with the Mann–Whitney *U*-test. The changes in proteinuria and urinary VEGF excretion were analyzed using two-tailed Wilcoxon's signed rank test. Simple linear regression analyses were used to study interrelationships between various parameters. The calculations were performed using GraphPad Prism™ (GraphPad Software, Inc., San Diego, CA, USA) software. *P* values of less than 0.05 were considered significant.

RESULTS

Urinary VEGF in patients and control subjects

The urinary VEGF excretion was 68 ± 10 (median 46; 95% CI, 49 to 88) ng/mmol U_{Cr} in normal control subjects (Fig. 1). Patients with MGN excreted 16 ± 3 (median 12; CI, 10 to 23) ng/mmol U_{Cr}, which was significantly less than the normal subjects ($P < 0.0001$, ANOVA). The excretion was 55 ± 14 (median 56; CI, 24 to 86) ng/mmol U_{Cr} in patients with minimal change nephropathy (P = NS compared with the normal subjects). In focal segmental glomerulosclerosis (FSGS), the excretion was elevated to 160 ± 29 (median 154; CI, 95 to 226) ng/mmol U_{Cr} ($P = 0.002$), and in necrotizing glomerulonephritis, the urinary VEGF excretion of 184 ± 68 (median 127; CI, 24 to 344) ng/mmol U_{Cr} was also elevated compared with the normal subjects ($P = 0.01$). In diabetic nephropathy, the urinary VEGF excretion was 101 ± 25 (median 88; CI, 45 to 156) ng/mmol U_{Cr} (P = NS).

The median serum VEGF concentration was 80 (range from 5.0 to 14,800) ng/L in patients with MGN, 60 (5 to 1,100) ng/L in minimal change nephropathy, 288 (5 to 11,250) ng/L in FSGS, 120 (5 to 20,000) ng/L in necrotizing glomerulonephritis, and 260 (20 to 3,500) ng/L in patients with diabetic nephropathy (P = NS, ANOVA).

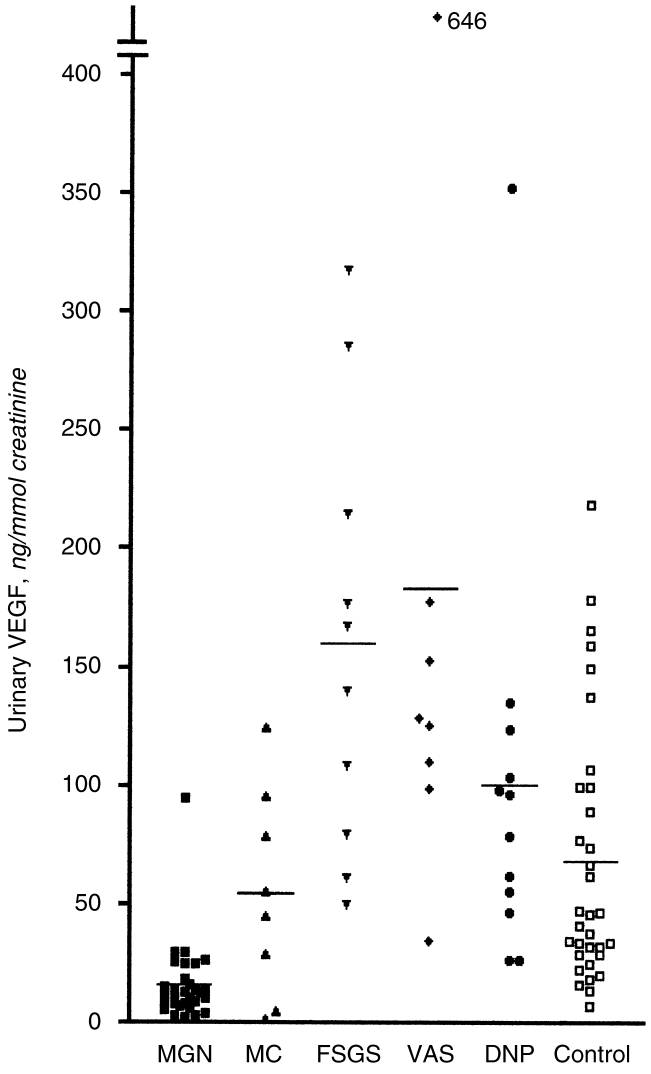

Fig. 1. Urinary vascular endothelial growth factor (VEGF) excretion in different glomerular diseases and healthy control subjects. Abbreviations are: MGN, membranous glomerulonephritis; MC, minimal change glomerulonephritis; FSGS, focal segmental glomerulosclerosis; VAS, vasculitis; DNP, diabetic nephropathy; Control, healthy controls. The lines indicate the mean values for each group.

Urinary VEGF did not correlate with albumin excretion ($r = -0.002$, P = NS), urinary α_1-microglobulin ($r = 0.03$, P = NS), S_{Cr} ($r = -0.2$, P = NS), or 24-hour creatinine clearance ($r = 0.28$, P = NS) in patients with MGN. There was no significant correlation to urinary TGF-β1 ($r = 0.02$, P = NS) or urinary TNF-α excretion ($r = 0.22$, P = NS). Moreover serum VEGF concentrations did not correlate with urinary VEGF ($r = -0.28$, P = NS) in patients with MGN or in any of the control groups with various glomerular diseases (data not shown).

Clinical activity of MGN and urinary VEGF

Associations between changes in the clinical activity of MGN (assessed by proteinuria) and urinary VEGF

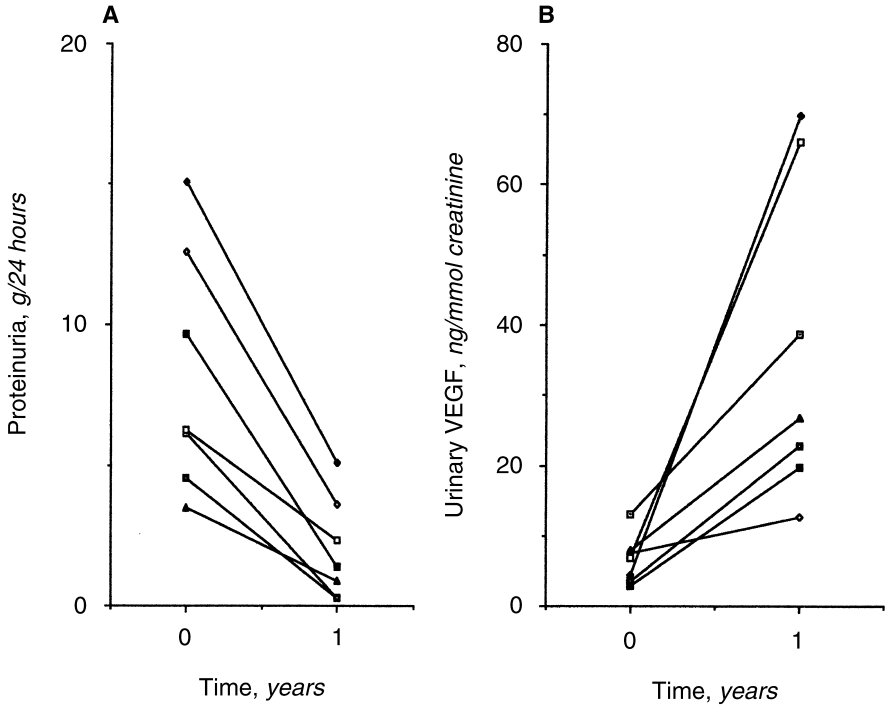

Fig. 2. Patients with improving membranous glomerulonephritis. Changes in urinary protein (*A*) and VEGF (*B*) excretion over one year.

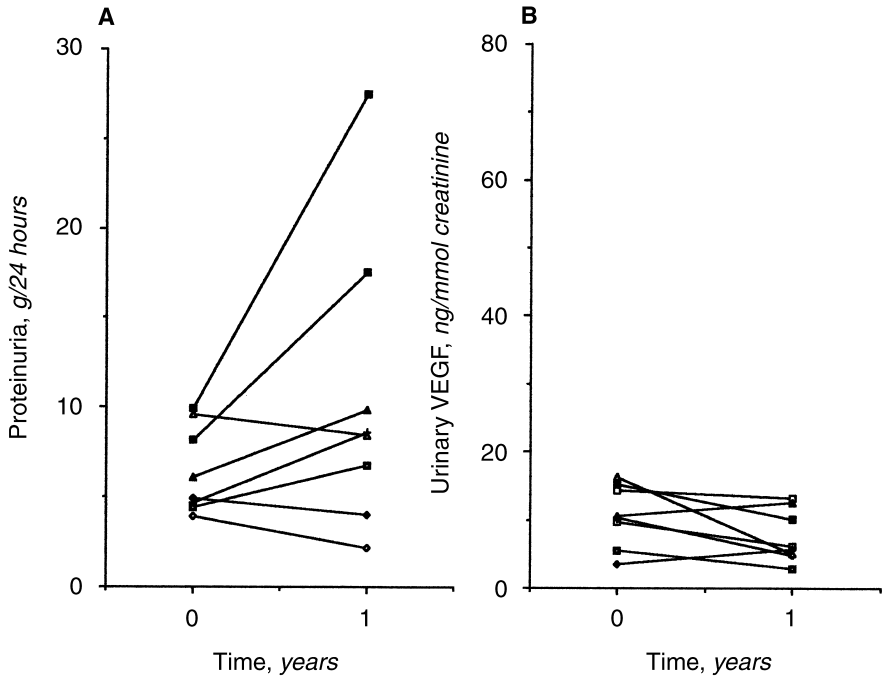

Fig. 3. Patients with persistent membranous glomerulonephritis. Changes in urinary protein (*A*) and VEGF (*B*) excretion over one year.

were studied in 15 patients presenting with the nephrotic syndrome. They were followed up for 12 (range, 10 to 14) months after the initial renal biopsy (that is, diagnosis of MGN). These patients were divided into two groups according to the evolution of proteinuria (Figs. 2 and 3).

Seven patients (Fig. 2) either entered remission or their urinary protein excretion decreased by at least 50% during the follow-up (improving MGN). In this group, proteinuria decreased from 8.2 ± 1.6 to 2.0 ± 0.7 g/24 h ($P = 0.016$), while the urinary VEGF excretion increased from 6.6 ± 1.3 to 36.2 ± 8.4 ng/mmol U_{Cr} ($P = 0.016$).

In eight patients (Fig. 3), the abundant urinary protein

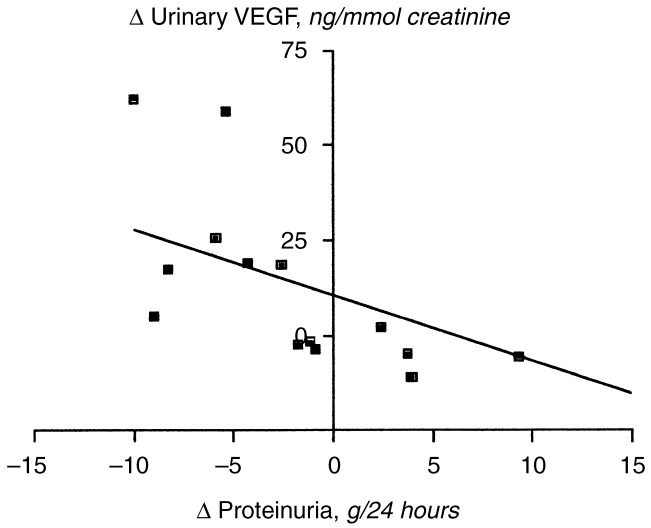

Fig. 4. Correlation between the change in urinary protein excretion (Δ proteinuria) and VEGF excretion (Δ urinary VEGF) during a one-year follow-up ($r = 0.57$; $P < 0.026$).

excretion either persisted or increased (persistent MGN). In this group, proteinuria was 6.5 ± 0.9 g/24 h initially and 10.6 ± 2.9 g/24 h at 12 months (P = NS). The urinary VEGF excretion was 10.7 ± 1.6 ng/mmol U_{Cr} initially and 7.6 ± 1.4 ng/mmol U_{Cr} at 12 months (P = NS).

Figure 4 shows the combined data of the 15 patients. There was a negative correlation between the change of proteinuria during the follow-up and the change in urinary VEGF excretion ($r = -0.57$, $P = 0.026$). Thus, as the activity of MGN decreased either spontaneously or as a result of therapy, the urinary VEGF excretion increased and vice versa.

DISCUSSION

In the present study, we examined 69 patients with a variety of glomerular diseases and 33 normal subjects. It appeared that the normal subjects had detectable urinary VEGF excretion, and compared with them, the excretion was unchanged in diabetic nephropathy and minimal change glomerulonephritis, but was elevated in patients with FSGS and necrotizing glomerulonephritis. In contrast, MGN was associated with significantly decreased urinary VEGF excretion, while decreasing clinical activity (proteinuria) was associated with increasing urinary VEGF excretion toward the levels observed in the healthy subjects.

Vascular endothelial growth factor has been suggested to have an essential role in angiogenesis, and it is also a central molecule in embryonic development, wound healing, and tumor growth [13, 28]. Its overexpression is observed in immunologic dermatologic diseases like psoriasis and pemphigoid [29, 30]. Glomerulogenesis is dependent on normal activity of VEGF, which may mediate communication between Bowman's capsule and capillary endothelial cells [31]. In normal fetal and adult kidneys, the most intense VEGF production is found in podocytes and collecting duct cells, and the mRNA for its receptors is seen in glomerular endothelia and peritubular capillaries [32]. Its physiological function in kidneys, as well as role in the pathogenesis of renal diseases, is, however, unclear.

Certain features of VEGF suggest that it might be involved in altered glomerular permeability and pathophysiology. It is a highly potent inducer of vascular permeability. It affects the chemotaxis of monocytes and activates proteases [13, 28]. In protein-overload nephrosis induced using bovine serum albumin, the glomerular expression of mRNA of VEGF and its receptors correlated with the severity of proteinuria [33], whereas the expression was profoundly down-regulated in experimental anti-glomerular basement membrane glomerulonephritis and puromycin aminonucleoside nephrosis (abstract; Tang et al, *J Am Soc Nephrol* 6:855, 1995). However, although the administration of VEGF to non-nephritic rats led to a reversible fall in blood pressure, it did not induce proteinuria or glomerular histologic changes [34]. Likewise, VEGF did not affect glomerular permeability for albumin in isolated perfused rat kidneys, but it enhanced the relaxation of renal vasculature after norepinephrine infusion, an effect dependent on nitric oxide [35]. In the experimental anti-Thy nephritis, VEGF may participate the healing of the lesions [36].

Some previous articles have focused on the expression of VEGF in human renal diseases. In the study of Gröne, Simon, and Gröne, VEGF mRNA and protein were expressed in proximal and distal tubular epithelia, particularly in conditions showing acute vascular obstruction such as acute vascular allograft rejection or necrotizing vasculitis [17]. Interestingly, in the present study, patients with necrotizing glomerulonephritis had elevated urinary VEGF excretion. This finding is in concord with a recent preliminary article in which elevated serum VEGF levels were observed in pauci-immune (perinuclear antineutrophil cytoplasmic antibody positive) rapidly progressive glomerulonephritis [37]. The up-regulated expression (and urinary excretion) of VEGF in vasculitis may be caused by hypoxia, which has previously been shown to induce VEGF gene and protein as well as receptor expression [38, 39].

Shulman et al studied 47 patients with a variety of renal diseases using immunohistochemistry and in situ hybridization [21]. They observed that VEGF expression was decreased or absent in sclerotic glomeruli, glomeruli compressed by crescents, glomeruli with a marked hypercellularity (systemic lupus erythematosus), and in areas occupied by matrix nodules (diabetic nephropathy). More specifically, the three patients with MGN showed

expression of VEGF in preserved glomeruli. In the congenital nephrotic syndrome of the Finnish type, no consistent changes in the production or expression of VEGF or its receptor KDR were found in the study of Haltia et al [18]. Mesangial VEGF expression has been reported to be characteristic for early lesions of human mesangial proliferative glomerulonephritis [20]. Webb et al studied steroid-sensitive nephrotic syndrome in childhood [34]. No alterations in serum concentration, peripheral blood mononuclear cell mRNA expression, or urinary excretion of VEGF were found during relapses. Interestingly, it was recently suggested that urine from patients with benign prostatic hyperplasia and prostate cancer increased capillary endothelial cell proliferation, while urinary VEGF excretion was increased in the former and decreased in the latter condition [40]. In contrast, urinary VEGF (from morning mid stream specimens) was elevated and correlated with tumor recurrence rate in patients with bladder cancer [41].

The available data on VEGF in MGN are thus scant, and to our knowledge no previous studies have used urinary assays of this molecule in various renal diseases. The assay used may not necessarily measure the intact functional VEGF molecule. Even if we, in part, are measuring nonfunctional VEGF or its degradation products, the assay still revealed a clear difference between MGN and other diseases studied. The significance of the decreased urinary VEGF excretion in our patients with MGN is speculative. This phenomenon was not observed in the other, pathogenetically different glomerular diseases causing abundant proteinuria apart from two patients with minimal change glomerulonephritis (Fig. 1). Furthermore, MGN seemed to differ markedly from FSGS in which the VEGF excretion was increased.

In MGN, the initial immunologic events affect podocytic foot processes under which immune deposits are formed [3]. This leads to activation of the complement cascade with formation of C5b-9, proteinuria, and production of TNF-α in visceral epithelial cells [5]. We showed previously that urinary TNF-α excretion was increased in active MGN, while this was not observed in diabetic nephropathy [42]. TNF-α activates capillary endothelium to express adhesion molecules [43] and in MGN urinary TNF-α excretion correlates with peritubular capillary expression of E-selectin [44]. On the other hand, TNF-α inhibits VEGF-induced endothelial cell proliferation and down-regulates its receptors in endothelial cells [45]. We did not, however, find significant correlation between urinary TNF-α and VEGF excretion.

In experimental models, VEGF expression is influenced by several growth factors and cytokines [28]. In cultured mesangial cells, TGF-β increases the expression of VEGF, while it is inhibited by dexamethasone [19]. In the present study, urinary VEGF did not correlate with urinary TGF-β1 proposed to be a marker of intrarenal fibrotic processes [25] or with α1-microglobulin, an indicator of proximal tubular cell injury [27].

Since the early immunologic processes in MGN affect glomerular podocytes, which in normal kidneys are the major site for production and expression of VEGF, it is possible that the podocyte injury results in their blunted capacity to secrete VEGF. As the clinical activity of the disease decreased either spontaneously or following immunomodulating therapy, a markedly increased urinary VEGF excretion was evident in our patients. This may hypothetically be explained by at least partial recovery of the podocyte injury in these patients. Further studies are warranted to show if the diminished urinary VEGF excretion is specific for active MGN. Moreover, serial determination of urinary VEGF may be more useful than single measurements in the assessment of the activity of MGN.

In conclusion, we used an enzyme immunoassay to study urinary VEGF excretion in a variety of glomerular diseases. The findings suggest for a decreased VEGF excretion in clinically active MGN but not in the other conditions studied. At follow-up, changes in protein excretion correlated inversely with changes in VEGF excretion. Hence, in patients with partial of full clinical remission (assessed by proteinuria), the excretion increased. Urinary VEGF may reflect the immunologic podocyte injury in MGN.

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