American Journal of Pathology, Vol. 146, No. 6, June 1995

Tumor Necrosis Factor-α Is Expressed by Glomerular Visceral Epithelial Cells in Human Membranous Nephropathy

T. James Neale, Beate M. Rüger, Heather Macaulay, P. Rod Dunbar, Qurratulain Hasan, Alice Bourke, Rosalind P. Murray-McIntosh, and A. Richard Kitching

From the Department of Medicine, Wellington School of Medicine, Wellington, New Zealand

The role of tumor necrosis factor alpha (TNF-α) was examined in biopsy-proven glomerulonephritis by immunohistochemistry, in situ hybridization, immunogold electron microscopy, immunoassay in serum and urine, and urinary immunoblot. Striking glomerular capillary wall and visceral glomerular epithelial cell TNF-α protein staining was observed in all cases of membranous nephropathy and membranous lupus nephropathy. Staining was less frequently observed in crescentic glomerulonephritis and in isolated cases of other histological subtypes of glomerulonephritis, usually in association with glomerular macrophages. By immunogold electron microscopy TNF-α was localized in membranous nephropathy within the visceral glomerular epithelial cells, and also in the glomerular basement membrane, especially in relation to immune deposits. In situ hybridization localized TNF-α mRNA exclusively to glomerular epithelial cells in all biopsies with membranous morphology but not in other histological subtypes. Concentrations of TNF-α were significantly increased compared with normal controls in the urine of patients with membranous nephropathy and with crescentic glomerulonephritis. The expression of TNF-α by glomerular epithelial cells exclusively and universally in biopsies showing a membranous morphology strongly suggests this cytokine has a role in the pathogenesis of membranous nephropathy. (Am J Pathol 1995, 146:1444–1454)

Tumor necrosis factor (TNF)-α, a 17-kd protein encoded in the major histocompatibility complex locus on chromosome 6, is produced by a wide range of cell types but predominantly by cells of the monocyte lineage.[1] It is an important regulator of immune responses, generally acting to promote inflammation and as such has a potential role in glomerulonephritis (GN). TNF-α induces specific transcription factors in target cells and stimulates production of other cytokines, particularly interleukin (IL)-1 and IL-6. Together with these cytokines, TNF-α production is associated with endotoxic shock, and the familiar systemic inflammatory syndrome of fever, cachexia, and acute phase response. TNF-α upregulates the expression of class I MHC molecules, vascular adhesion molecules, and procoagulant activity, amplifying local inflammatory responses. Other effects of TNF-α of relevance to GN include the promotion of angiogenesis and fibrosis and a complex interaction with oxygen radical metabolism.

Possible sources of TNF-α in human GN are intrinsic glomerular cells and extrinsic inflammatory cells recruited into the glomerulus. Previous studies of TNF-α production by normal human kidney cells have produced conflicting results. Several groups found no detectable TNF-α mRNA or protein in normal human kidney tissue,[2–4] although others reported detectable TNF-α mRNA[5,6] and mesangial staining with a polyclonal antibody.[6] Rat mesangial cells can synthesize TNF-α,[7,8] but production of TNF-α by human mesangial cells in tissue culture could not be demonstrated in one published study, although these cells did secrete IL-1.[9] Production of TNF-α by cultured rat glomerular epithelial cells (GECs) has recently been reported.[10]

In studies of human renal disease to date, TNF-α has been associated predominantly with extrinsic in-

Supported by the Health Research Council of New Zealand, the Wellington Medical Research Foundation, the Research Trust Funds of the Wellington Area Health Board, and Glaxo New Zealand, Ltd.

Accepted for publication March 1, 1995.

Address reprint requests to B. M. Rüger, Department of Medicine, Wellington School of Medicine, PO Box 7343, Wellington, New Zealand.

flammatory cells. In biopsies obtained from patients with immunoglobulin (Ig)A nephropathy, cells expressing TNF-α have been identified as predominantly monocytes/macrophages.[11] In tissue from patients with anti-neutrophil cytoplasmic antigen-positive GN, TNF-α expression was found in several sites, predominantly in infiltrating mononuclear cells and within crescents, although the parietal epithelial cells and some tubular cells stained for TNF-α.[3]

Similarly, in experimental renal disease, mononuclear cells have been considered to be the major source of TNF-α. TNF-α synthesis by infiltrating macrophages has been demonstrated in mouse models of lupus nephritis,[12,13] in acute aminonucleoside nephrosis in rats,[12,14] and in anti-glomerular basement membrane (GBM) antibody-induced GN in rabbits.[15] In the latter model, synthesis of TNF-α by intrinsic glomerular cells was reported as quantitatively insignificant.[15]

We studied the expression of TNF-α mRNA and protein in 72 renal biopsy specimens from patients with a range of glomerular pathological diagnoses. In addition to detecting TNF-α synthesis in previously reported sites particularly in macrophages within crescentic and IgA nephropathy biopsies, we have demonstrated for the first time expression of TNF-α by glomerular visceral epithelial cells. TNF-α synthesis by GECs was strictly limited to cases with a membranous morphology. The importance of our finding was supported by urinary immunoblotting and immunoassay, as patients with membranous nephropathy (MN) had significantly elevated urinary TNF-α concentrations compared with normal subjects.

Materials and Methods

Samples

Archival renal biopsy tissue was available from 72 patients with GN and from 3 patients without diagnostic abnormalities on light microscopy. Serum and urine samples were collected from 60 patients with biopsy-proven GN and from 20 healthy volunteers. Samples were stored frozen at −70 C in 1-ml aliquots. All subjects gave informed written consent, and the study was approved by the Ethical Committee of the Wellington Area Health Board. Numbers of patients in each GN sub-group are shown in Table 1.

Immunohistochemistry

Sections of formalin-fixed, paraffin-embedded tissue were dewaxed with xylene, rehydrated to Tris-buffered saline (TBS), pH 7.5, and digested with bacterial protease XXIV (Sigma Chemical Co., St Louis, MO) for 10 minutes at 37 C. The slides were incubated overnight at 4 C with a monoclonal antibody to human TNF-α (clone 199, IgGκ; Boehringer-Mannheim, Mannheim, Germany) diluted 1:50 in Tris-buffered saline/1% bovine serum albumin. Endogenous peroxidase was inhibited with 0.3% H_2O_2 in Tris-buffered saline for 10 minutes at room temperature. Biotinylated anti-mouse Igs were applied for 30 minutes at room temperature followed by peroxidase-conjugated streptavidin for 30 minutes at room temperature (SuperSensitive system, BioGenex, San Ramon, CA). The substrate diaminobenzidine was added until the desired staining intensity occurred. After counterstaining with hematoxylin, the sections were dehydrated and mounted in DPX.

Control experiments were conducted on identically prepared sections as follows. For primary antibody substitution, the primary antibody was omitted or substituted with anti-Mac-387 (Dako Corp., Carpinteria, CA), a monoclonal antibody of the same class (IgG1κ), which stains monocytes/macrophages. For specific adsorption of anti-TNF-α antibody reactivity, 2 μg/ml anti-TNF-α and 1 ng/ml TNF-α (NIBSC, London, UK) were mixed at room temperature for 4 hours and then centrifuged at 10,000 rpm before applying the supernatant as primary antibody. For staining for IgG, human IgG was revealed with monoclonal mouse anti-human IgG antibody (Dako) and the SuperSensitive detection system.

In Situ *Hybridization*

Oligonucleotide probes (Table 2) based on the coding region of the published human TNF-α sequence[16] were purchased from Operon Technologies (Alameda, CA). All probes were 3′-end labeled with digoxigenin by using the Boehringer-Mannheim oligonucleotide tailing kit. The manufacturer's instructions were followed, except that the labeling reaction time was reduced to 10 minutes to minimize probe digestion,[17] and the final ethanol wash was omitted. Formalin-fixed paraffin sections were prepared for hybridization as previously reported,[18] except that digestion with 1.5 μg/ml proteinase K (Boehringer-Mannheim) was preceded by dehydration through graded ethanol, and post-fixation was followed by a 10-minute incubation in 0.25% acetic anhydride in triethanolamine. Control slides were incubated with 100 μg/ml RNAse A (Boehringer-Mannheim) for 2 hours at 37 C. The hybridization buffer contained 600 mmol/L sodium chloride, 50 mmol/L Tris HCl, pH 7.5, 1% sodium pyrophosphate, 5 mmol/L EDTA, 10% formamide, 5%

Table 1. *Subjects Studied*

Diagnosis	Urine (*n*)	Serum (*n*)	IHC (*n*)	ISH (*n*)
Crescentic GN	3	3	11	5
Focal GN	7	6	7	
Focal glomerulosclerosis	11	9	10	5
IgA nephropathy	8	8	8	5
Membranous nephropathy	6	3	10	10
Mesangiocapillary GN	3	3	3	
Minimal change disease	5	5	5	5
Mesangioproliferative GN	5	4	5	
Lupus nephritis	12	12	13	7
Lupus nephritis of membranous type	1	1	3	3
Total GNs	60	53	72	37
Normal	12	20	3	3

Numbers of subjects in each GN subgroup studied by TNF-α immunoassay of urine and serum and by immunohistochemistry (*IHC*) and *in situ* hybridization (ISH) for TNF-α protein and mRNA.

PEG 6000, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, and 160 ng/μl sheared salmon sperm DNA. Prehybridization for 1 hour at 37 C was followed by overnight hybridization at 42 C with 200 ng of either the anti-sense probes, in an equimolar mixture, or the sense probe. Slides were rinsed in 2X standard saline citrate (SSC), then washed once with 0.2X SSC for 30 minutes, twice with 0.1X SSC for 30 minutes, at 22 C, and once with 0.1X SSC for 30 minutes at 37 C. Hybridized probe was detected with alkaline phosphatase-labeled anti-digoxigenin antibody, and nitroblue tetrazolium/X-phosphate substrate according to the manufacturer's instructions (Boehringer-Mannheim). Slides were counterstained with neutral red, air dried, and mounted in DPX.

Immunogold Electron Microscopy

Renal biopsy material was fixed in periodate-lysine paraformaldehyde and embedded in LR white resin as previously described,[19,20] and ultra-thin sections were prepared on gold grids. Sections were incubated with 0.01 mol/L glycine for 10 minutes, followed by monoclonal anti-TNF-α antibody (Boehringer Mannheim), 1:50, in phosphate-buffered saline/0.1% bovine serum albumin/1% Tween-20 for 5 hours. Rabbit anti-mouse IgG (Dako) was applied for 1 hour, followed by gold-labeled goat anti-rabbit antibody (10-nm particle size, Amersham, Buckinghamshire, UK) for 5 hours. All incubations were performed at room temperature and were followed by extensive washes with 0.01 mol/L phosphate-buffered saline. After a final wash in ultra-pure water, sections were counterstained with uranyl acetate for 5 minutes. Preparations were viewed in a Zeiss 109 electron microscope.

Extensive specificity controls were performed, including omission and adsorption of the primary antibody, as described above for immunohistochemistry. In addition, monoclonal anti-vimentin antibody, 1:100 (Dako), was substituted for the primary antibody to reveal intermediate filaments in the cytoplasm of GECs as a marker of that cell type.

Immunoassay

A commercial immunoradiometric assay was used according to the manufacturer's instructions (Medgenix, Fleurius, Belgium), except that aliquots of the TNF-α protein standards provided (5000, 1500, and 15 pg/ml) were diluted to generate additional standards (1000, 750, and 10 pg/ml). The sensitivity of this assay as defined by the mean of the zero standard plus two standard deviations was 5 pg/ml. All assay results were calibrated with standard concentrations of TNF-α obtained from the UK National Institute of Biological Standards and Controls. The immunoradiometric assay did not detect TNF-β, IL-1, IL-2, or interferon-γ. Inter-assay variation was 9.8% and intra-assay variation was 6.9%. Data were processed with curve interpolation software that used a four-parameter immunoassay model.[21]

Table 2. *Oligonucleotides Used in* Situ *Hybridization*

	Sequence	Bases[16]
Anti-sense	5′-ACCGCCTGGAGCCCTGGGGCCCCCC-3′	240–216
	5′-GGGGAACTCTTCCCTCTGGGGGCCG-3′	344–320
	5′-GGCCTCAGCCCCCTCTGGGGTCTCC-3′	713–689
	5′-CCTTCTCCAGCTGGAAGACCCCTCC-3′	765–741
Sense	5′-CAGAGGGAAGAGTTCCCCAG-3′	327–346

Immunoblotting of Urine

Sodium dodecyl sulfate polyacrylamide gel electrophoresis of 30 μl from patient urine samples (concentrated 5X; Minicon, Millipore, Bedford, MA) and TNF-α standards (10 ng; UK National Institute of Biological Standards and Controls) was performed on 10% gels according to the procedure of Laemmli.[22] Proteins were visualized by silver staining and then transferred to Immobilon (Millipore) membranes in a Transblot semi-dry cell (Bio-Rad, Hercules, CA) at 15 V for 25 minutes. The membrane was blocked in phosphate-buffered saline/0.5% bovine serum albumin for 30 minutes before incubation with anti-human TNF-α monoclonal antibody (Boehringer-Mannheim), 1:50, for 2 hours. The membrane was washed in phosphate-buffered saline/0.05% Tween-20 and then exposed sequentially to biotinylated anti-mouse Ig (BRL/Gibco, Bethesda, MD), 1:3000, and streptavidin-biotin-peroxidase complex (Amersham), 1:2000. Bound antibody was visualized by incubation in enhanced chemiluminescence substrate (Amersham) for 60 seconds and exposure to photographic film (X-Omat K, Kodak) for 15 seconds.

Statistics

Quantitative data were compared by the nonparametric Mann-Whitney *U*-test (for two-group comparisons) and the Kruskal-Wallis test (for multi-group analysis). *P* values greater than 0.05 were considered not significant and are not cited in the text.

Results

Immunohistochemistry

Staining for TNF-α was observed in GECs and the GBM of all biopsies (10/10) obtained from patients with MN (Figure 1A, C). Identical staining was observed in all cases (3/3) of lupus nephritis with membranous morphology (Figure 2A) but not in any lupus nephritis biopsy tissue of nonmembranous type (0/10). This staining did not occur when the anti-TNF-α antibody was pre-adsorbed with TNF-α (Figure 2C). Macrophage/monocyte staining with Mac-387 was very rarely observed in any membranous biopsy tissue (less than 1 cell per 10 glomeruli). All biopsies showing membranous histology were graded between stages I and III[23]; intensity of GEC staining for TNF-α did not directly correlate with stage of disease.

In nonmembranous biopsies (n = 59), including those with prominent IgG deposition, TNF-α was not detected in GECs or the GBM (Figure 2D). In the majority of cases of crescentic GN, mononuclear cells in the crescents stained for TNF-α, as previously reported by others. This staining co-localized with Mac-387 (not shown). In some cases of IgA nephropathy (4/8), mesangial staining for TNF-α was observed, predominantly localized to mononuclear cells. Tubular staining for TNF-α was observed in many biopsies of all histological subtypes.

In Situ *Hybridization*

In all cases of MN (10/10) and lupus nephritis with membranous morphology (3/3), TNF-α mRNA was localized to GECs (Figures 1B, D and 2B). Binding of the anti-sense probes to these cells was prevented by preincubation with RNAse, and sense probes did not produce a positive signal. No other biopsy examined showed any labeling of GECs (minimal change disease, focal glomerulosclerosis, IgA nephropathy, crescentic, and other immune complex proliferative GN). In all biopsies from patients with crescentic GN, TNF-α mRNA was demonstrated in mononuclear cells located in crescents. Tubular TNF-α mRNA was detected in all cases with TNF-α protein expression in tubules.

Immunogold Electron Microscopy

Immunogold labeling for TNF-α was seen in cases of membranous morphology only, over GECs and in the GBM, especially over immune deposits (Figure 3). Preincubation of the anti-TNF-α antibody with TNF-α entirely abrogated this staining, and no specific labeling was observed when the primary antibody was omitted.

Measurement of TNF-α in Urine

Urine TNF-α concentrations are plotted as a scattergram in Figure 4. There was no significant difference between urinary TNF-α concentrations measured in control subjects and those in the total group of GN patients. Two GN patient subgroups showed urine TNF-α concentrations significantly higher than those of control subjects (patients with MN, $P = 0.027$; and with crescentic GN, $P = 0.009$). The majority of the samples from these patients had TNF-α concentrations above the highest control value. The single systemic lupus erythematosus patient with a urinary TNF-α concentration in excess of the normal range showed a membranous morphology on biopsy.

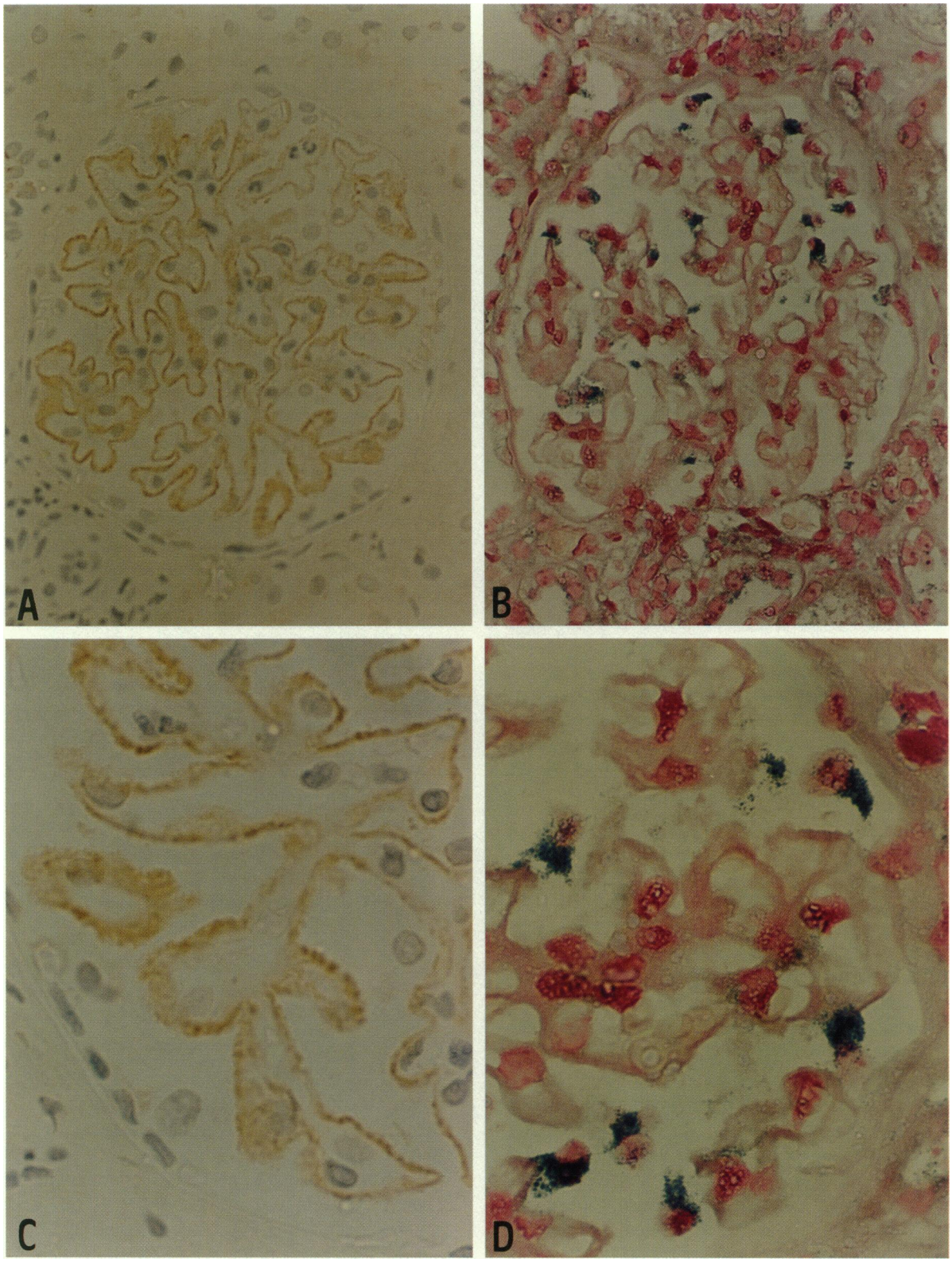

Figure 1. *TNF-α expression by visceral GECs in MN. Detection of TNF-α protein by immunohistochemistry* (A *and* C) *and TNF-α mRNA by* in situ *hybridization* (B *and* D) *in human membranous nephropathy. TNF-α protein is evident within the cytoplasm of visceral GECs (podocytes) and throughout the GBM, particularly in relation to immune deposits* (A *and* C). *TNF-α mRNA is intensely labeled (blue reaction product) with specific digoxigenin-labeled synthetic oligonucleotides exclusively within visceral GEC cytoplasm in MN. Magnification,* A *and* B, ×350; C *and* D, ×875.

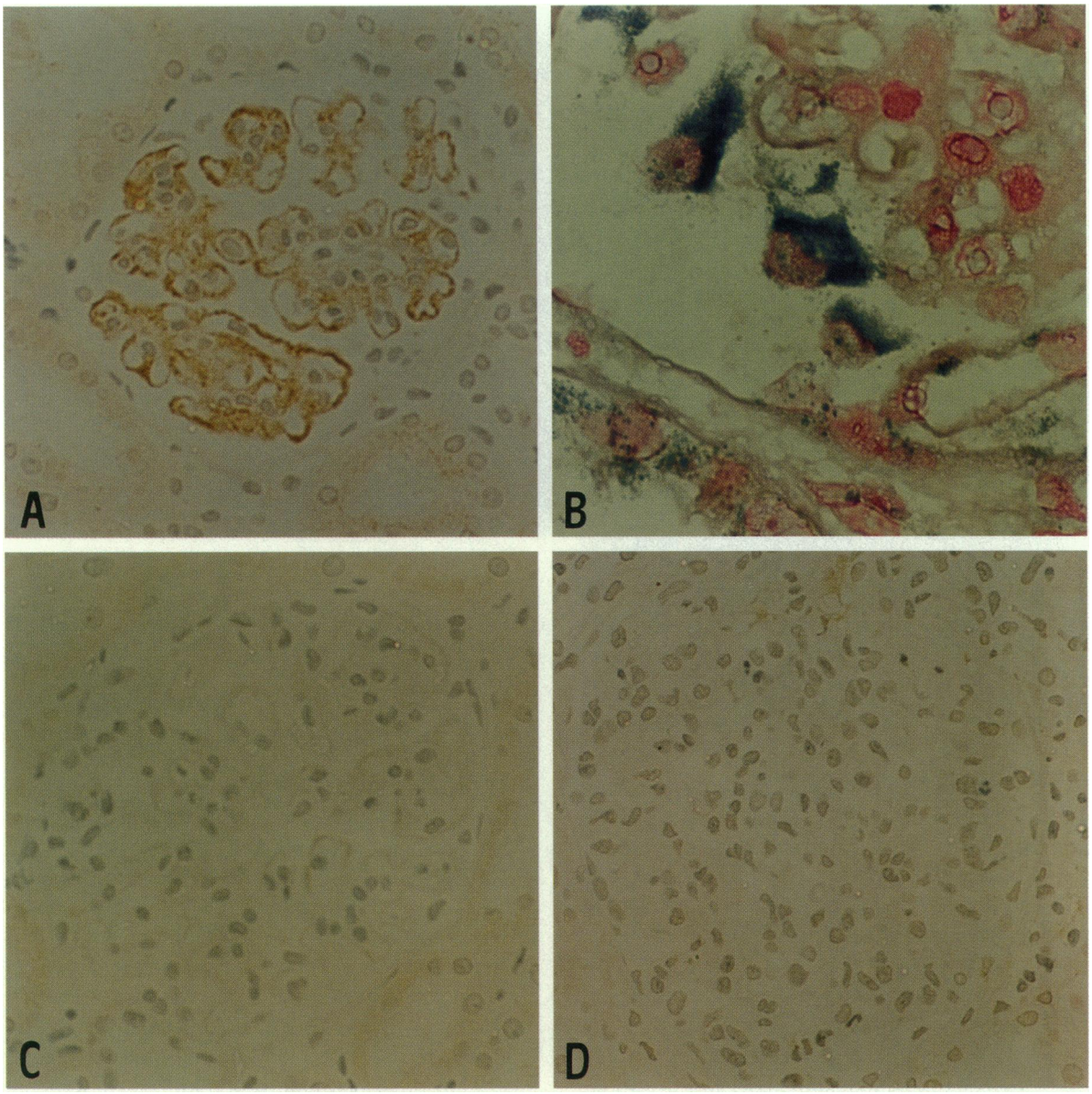

Figure 2. *TNF-α expression by visceral GECs in lupus nephropathy of membranous morphology. Detection of TNF-α protein by immunohistochemistry* (**A**) *and TNF-α mRNA by* in situ *hybridization* (**B**) *in the membranous histological variant of lupus nephropathy. TNF-α is prominent within visceral GECs and throughout the GBM* (**A**), *and TNF-α mRNA is intensely labeled (blue reaction product) exclusively within visceral GEC cytoplasm* (**B**). *Adsorption of anti-TNF-α monoclonal antibody with human TNF-α protein* (**C**) *abolished the reactivity seen in* (**A**), *confirming the antibody's specificity for TNF-α. No TNF-α protein was revealed within the glomeruli of a patient with post-streptococcal glomerulonephritis* (**D**) *in which Ig deposition was prominent, indicating that the TNF-α antibody did not bind to human Ig. Magnification,* **A**, **C**, *and* **D**, *×350;* **B**, *×875.*

Immunoblotting of Urine for TNF-α

Urine samples with elevated concentrations of TNF-α demonstrated bands of approximate molecular sizes 17 or 51 kd, representing monomer and trimer TNF-α, when immunoblotted with monoclonal anti-TNF-α (Figure 5). These bands were absent from all normal control urine specimens, but after addition of TNF-α antigen as a positive control, the 17-kd band could be detected (Figure 5).

Measurement of TNF-α in Serum

Serum TNF-α concentrations are plotted as a scattergram in Figure 6. There was a significant difference between serum TNF-α concentrations in control subjects and GN patients as a group ($P = 0.001$). The statistical significance of the variability in TNF-α between all of the subgroups, including controls, was confirmed by the Kruskal-Wallis test ($P = 0.012$). GN subgroups with TNF-α concentrations distributed sig-

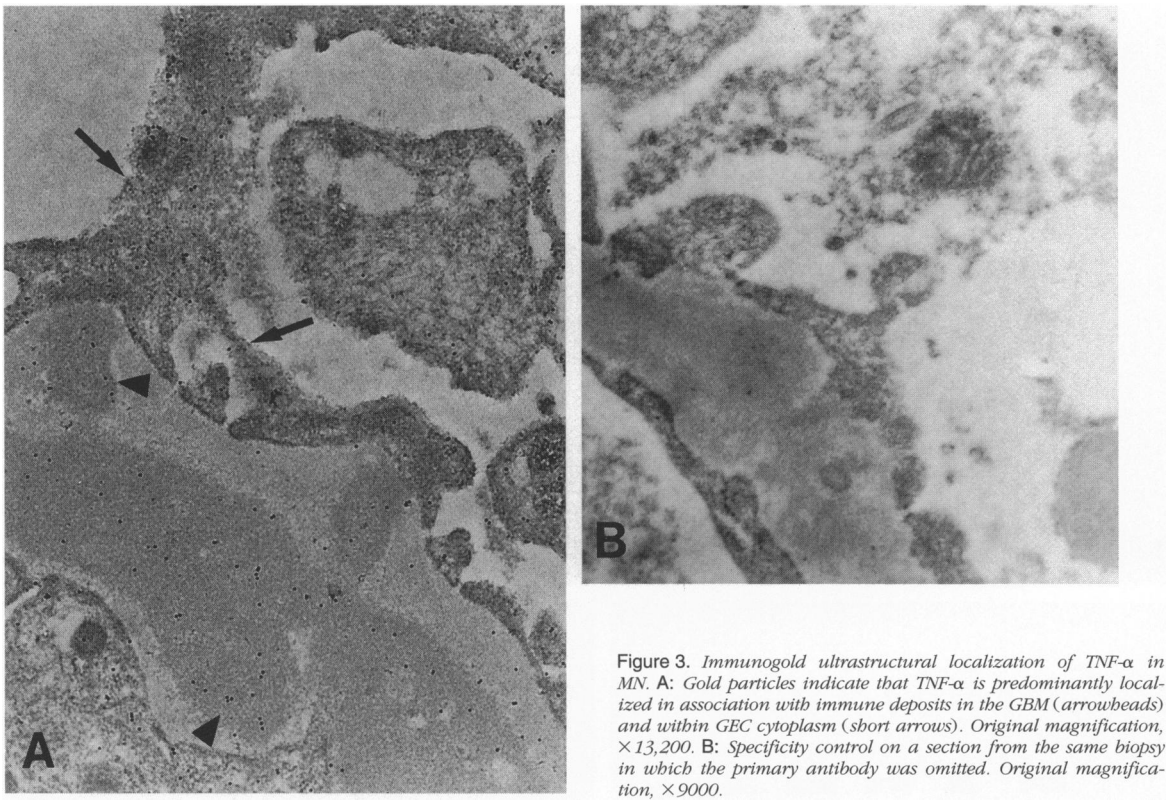

Figure 3. *Immunogold ultrastructural localization of TNF-α in MN.* A: *Gold particles indicate that TNF-α is predominantly localized in association with immune deposits in the GBM (arrowheads) and within GEC cytoplasm (short arrows). Original magnification, ×13,200.* B: *Specificity control on a section from the same biopsy in which the primary antibody was omitted. Original magnification, ×9000.*

nificantly higher than the control group were patients with IgA nephropathy (P = 0.006) and lupus nephropathy (P = 0.022). However, only one patient, with a biopsy diagnosis of type I mesangiocapillary GN, had a serum TNF-α concentration greater than the highest control value.

Discussion

We investigated the role of TNF-α in human GN using specific immunohistological and *in situ* hybridization methods and found unexpectedly that TNF-α is expressed by the visceral GECs of patients with membranous morphology, both of primary idiopathic and of lupus origin. In all membranous cases examined, TNF-α synthesis was confined to the GECs, with no evidence of TNF-α production by other intrinsic glomerular cells. Infiltrating leukocytes, another potential source of TNF-α production, were seldom seen in glomeruli with membranous histology. This pattern of TNF-α expression was not found in any other GN subtype and corresponded with significantly elevated concentrations of TNF-α in the urine. We did not observe a corresponding increase in serum TNF-α concentrations in the MN group, in contrast to one previous report.[24]

Our data from subjects with nonmembranous morphology on biopsy were substantially in agreement with previous observations.[3,11] In crescentic GN, TNF-α was detectable in monocyte/macrophages in crescents, and this correlated with elevated urinary TNF-α concentrations. In IgA nephropathy, TNF-α production was demonstrated occasionally within mesangial mononuclear cells. We also observed staining for TNF-α within tubules in many of the biopsies, and secretion of TNF-α by proximal tubular cells has previously been reported.[25,26] We were not able to detect TNF-α protein or mRNA in normal human renal tissue. Conflicting reports on this issue to date may relate to differences in methodology. Neither could we confirm the previously reported elevation in TNF-α concentrations in urine and serum from patients with focal glomerulosclerosis.[24] Our histopathological studies revealed no TNF-α expression in the kidneys of patients with focal glomerulosclerosis. However, patients with lupus nephropathy or IgA nephropathy had significantly higher serum TNF-α concentrations than controls, although none of these patients had values outside the normal range. It has previously been reported that cultured peripheral blood monocytes from patients with IgA nephropathy secrete greater amounts of TNF-α than control cultures.[27]

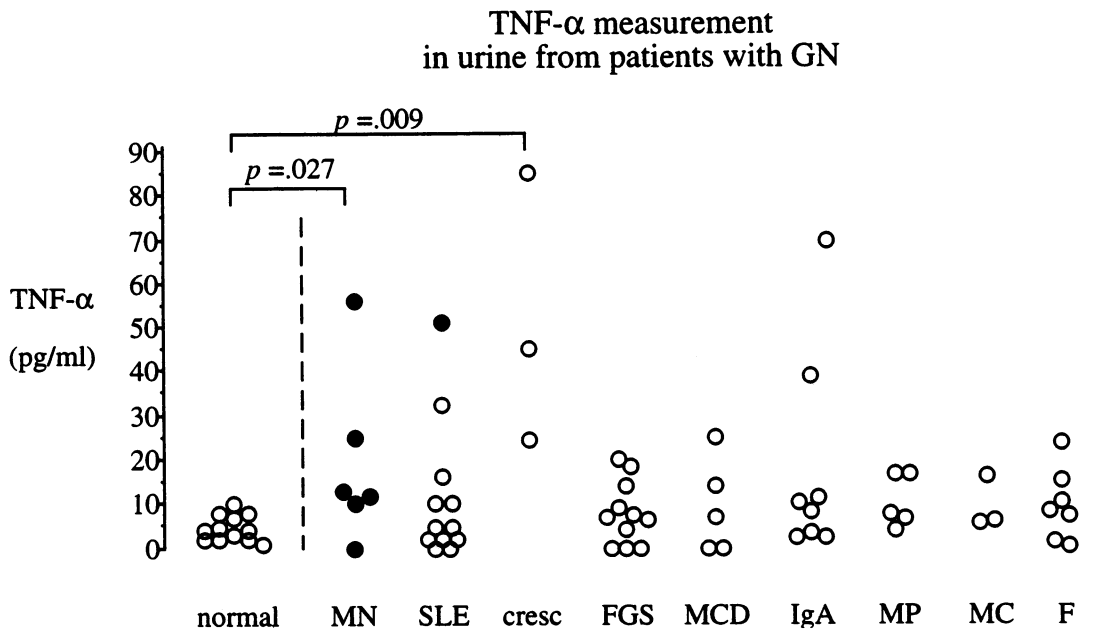

Figure 4. *TNF-α measurement in urine from patients with GN. MN, membranous nephropathy; SLE, systemic lupus erythematosus; cresc, crescentic GN; FGS, focal glomerulosclerosis; MCD, minimal change disease; IgA, IgA nephropathy; MP, mesangioproliferative GN; MC, mesangiocapillary GN; F, focal GN.*

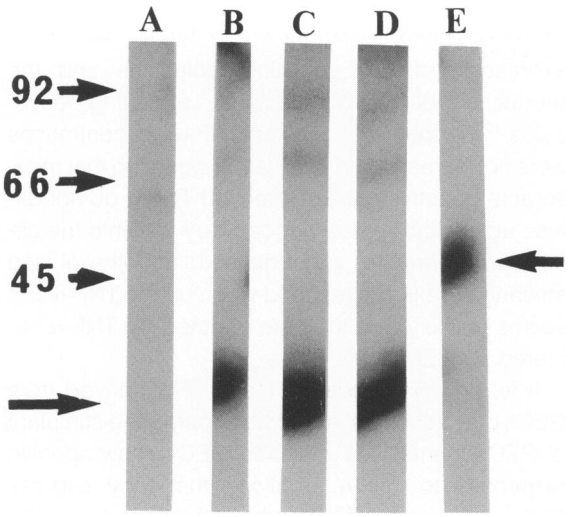

Figure 5. *Immunoblot of urine samples from patients with MN.* Lane A: *Molecular weight markers.* Lane B: *TNF-α antigen added to normal urine.* Lanes C, D, *and* E: *Urine from patients with MN. Left hand arrow indicates an approximately 17-kd band and right hand arrow an approximately 51-kd TNF-α trimer.*

The specific production of TNF-α by GEC in MN is of particular importance as these cells are regarded as being central to the pathogenesis of MN.[28] Furthermore, two other GN subtypes in which GECs show injury, focal glomerulosclerosis and minimal change disease, did not show TNF-α expression by GECs. It seems likely, therefore, that TNF-α expression by GECs is intimately linked with the pathogenetic events specific to MN. Human MN is increasingly believed to involve the binding of auto-antibody to GEC membrane proteins, on the basis of evidence from animal models.[29–33] The TNF-α synthesis by GECs demonstrated in this report may be a direct or indirect result of GEC perturbation by antibody.

Although the current report is the first to document expression of TNF-α by human visceral GECs in health or disease, recent studies in experimental animals confirm that GECs can express TNF-α and that intraglomerular TNF-α production is associated with proteinuria and disease progression.[10] Cultured rat GECs release TNF-α when exposed to adriamycin, aminonucleoside of puromycin, lipopolysaccharide, or platelet-activating factor,[10] suggesting that a range of noxious stimuli might induce release of TNF-α from GECs in humans.

Stimulation of GECs by complement might induce TNF-α expression. Complement deposition is a feature of MN and is presumed to be necessary to its pathogenesis.[28] Stimulation of cultured human mesangial cells with terminal complement proteins induces release of TNF-α,[34] although similar experiments have not been performed with GECs. In experimental MN (passive Heymann's nephritis), we have shown that antibody binding to GECs induces complement-dependent production of H_2O_2, by upregulating the respiratory burst NADPH oxidoreductase in GECs.[33,35] Reactive oxygen species modulate TNF-α release, and TNF-α may induce generation of reactive oxygen species.[12] It is possible

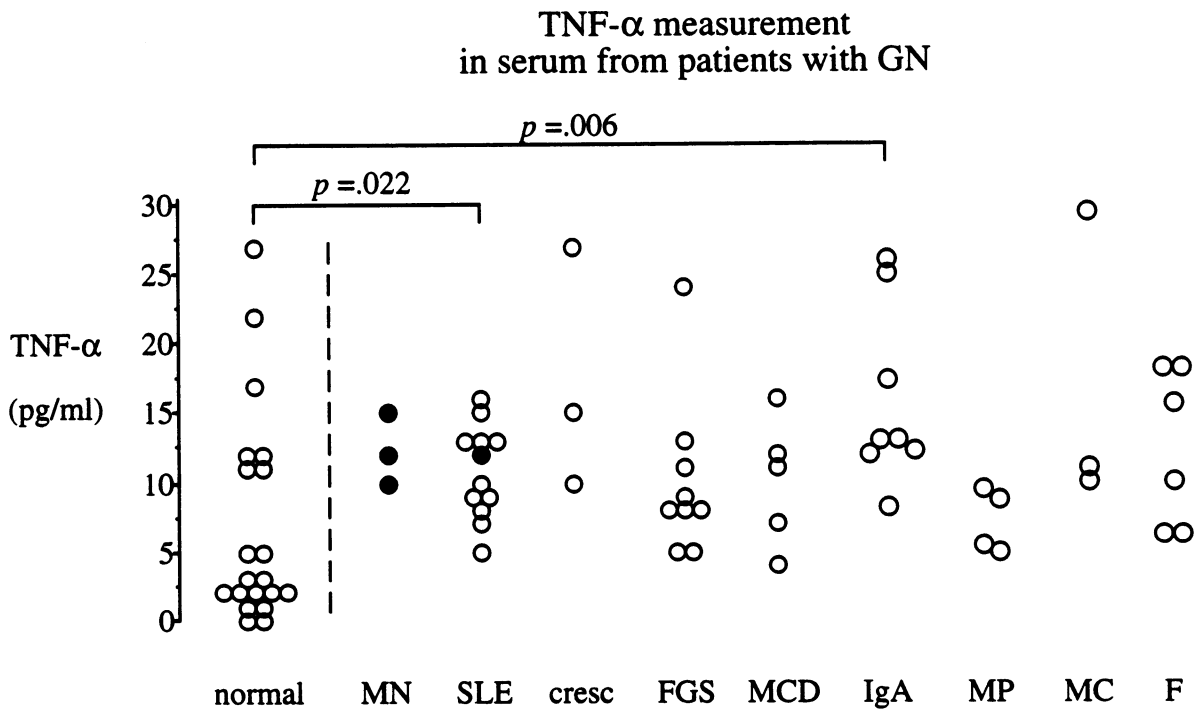

Figure 6. *TNF-α measurement in serum from patients with GN. For abbreviations see Figure 4.*

that antibody binding in MN leads to stimulation of GECs with activated complement products, upregulating GEC production of reactive oxygen species and TNF-α, which reinforce each other's release. Experiments to establish whether complement products can induce TNF-α synthesis in GECs will be of great interest. It is pertinent in this context that cultured human GECs have been reported to synthesize C3.[36]

The likely role of TNF-α expression by GECs in the pathogenesis of MN remains unclear. Adverse effects of TNF-α on glomerular function have been demonstrated in some experiments with animal models[10,12,37–40] but not in others.[41,42] TNF-α is directly cytotoxic to many glomerular cell types[43] and can promote procoagulant activity with formation of microthrombi[12] that could contribute to the renal vein thrombosis associated with MN. However, it is speculative whether GEC-derived TNF-α could reach endothelial cells or the mesangium by diffusion retrograde to plasma flow. Our immunogold electron microscopy data, which revealed prominent TNF-α staining in the GBM, particularly in relation to immune deposits, suggests TNF-α secreted by GECs in MN may infiltrate the GBM. One observation that mitigates against TNF-α reaching the endothelium is the lack of glomerular inflammatory cell recruitment in MN. Access of sufficient TNF-α to the glomerular endothelium and mesangium would be expected to upregulate the expression of cell adhesion molecules and the production of chemoattractants, attracting leukocytes.[12] Furthermore, plasma TNF-α concentrations were not increased in our study, suggesting that measurable quantities of GEC-derived TNF-α do not diffuse across the glomerular capillary wall into the circulation. Therefore, although both endothelial and mesangial cells can respond vigorously to TNF-α,[12] it seems unlikely that they are affected by TNF-α secreted by GECs in MN.

It is, however, possible that TNF-α derived from GECs may act as an autocrine or paracrine stimulant of GECs themselves. Cultured GECs show specific responses to TNF-α, implying that they express TNF-α receptors.[36,43] Possible effects of this autocrine stimulation include direct cytotoxicity,[43] increased secretion of metalloproteinases[44] that could degrade GBM,[45] and induction of reactive oxygen species.[46,47] Clearly human GEC receptor expression now needs to be studied *in vivo*.

Another possibility is that the majority of the TNF-α expressed by GECs remains membrane bound and is not released in sufficient quantities to activate other glomerular cells or GECs themselves. Membrane expression of TNF-α without substantial release of soluble TNF-α has been observed on many other cell types, including mesangial cells.[7] However, our urine data suggest that TNF-α is being released from GEC membranes in MN in significant quantities.

It is interesting to consider the time over which GECs must synthesize TNF-α in MN to provide the results obtained. All MN patients studied were biopsied within weeks of nephrological referral, but, as time of presentation depends on symptom severity and patient tolerance, biopsy will have been performed at very variable intervals after induction of disease. Despite this heterogeneity and the varied stages of disease seen on biopsy (from stages I to III), TNF-α synthesis was seen within the GECs of every case examined. We have not been able to study advanced cases of MN or cases in clinical remission, but our current data indicate that TNF-α production by GECs is a universal and constant feature of MN for at least the first few months of disease. Additional work is required to establish whether this GEC production of TNF-α correlates directly with the presence of clinical disease and to define its precise role in the pathogenesis of MN.

References

1. Tracey KJ, Cerami A: Tumor necrosis factor: a pleiotropic cytokine and therapeutic target. Annu Rev Med 1994, 45:491–503
2. Aikawa A, McLaughlin PJ, McDicken IW, Davies HM, Southern SA, Johnson PM, Bakran A, Sells RA: TNF staining of graft biopsies in renal transplantation. Transplantation 1993, 56:231–233
3. Noronha IL, Kruger C, Andrassy K, Ritz E, Waldherr R: *In situ* production of TNF-α, IL-1β and IL-2R in ANCA-positive glomerulonephritis. Kidney Int 1993, 43:682–692
4. Noronha IL, Hartley B, Cameron JS, Waldherr R: Detection of IL-1β and TNF-α message and protein in renal allograft biopsies. Transplantation 1993, 56:1026–1029
5. Tovey MG, Content J, Gresser I, Gugenheim J, Blanchard B, Guymarho J, Poupart P, Gigou M, Shaw A, Fiers W: Genes for IFN-β2 (IL-6), tumor necrosis factor, and IL-1 are expressed at high levels in the organs of normal individuals. J Immunol 1988, 141:3106–3110
6. Gogusev J, Augusti M, Chretien Y, Droz D: Interleukin-6 and TNF-α production in human renal cell carcinoma. Kidney Int 1993, 44:585–592
7. Affres H, Perez J, Hagege J, Fouqueray B, Kornprobst M, Ardaillou R, Baud L: Desferrioxamine regulates tumor necrosis factor release in mesangial cells. Kidney Int 1991, 39:822–830
8. Baud L, Oudinet JP, Bens M, Noe L, Peraldi M, Rondeau E, Etienne J, Ardaillou R: Production of tumor necrosis factor by rat mesangial cells in response to bacterial lipopolysaccharide. Kidney Int 1989, 35: 1111–1118
9. Abbott F, Tam FW, Ryan JJ, Rees AJ: Human mesangial cells synthesize interleukin 1α but not interleukin 1β, interleukin 1 receptor antagonist, or tumour necrosis factor. Nephrol Dial Transplant 1992, 7:997–1001
10. Gomez-Chiarri M, Ortiz A, Lerma JL, Lopez-Armada MJ, Mampaso F, Gonzalez E, Egido J: Involvement of tumour necrosis factor and platelet-activating factor in the pathogenesis of experimental nephrosis in rats. Lab Invest 1994, 70:449–459
11. Yoshioka K, Takemura T, Murakami K, Okada M, Yagi K, Miyazato H, Matsushima K, Maki S: *In situ* expression of cytokines in IgA nephritis. Kidney Int 1993, 44: 825–833
12. Baud L, Ardaillou R: Tumor necrosis factorα in glomerular injury. Kidney Int 1994, 45(Suppl)45:S32–S36
13. Boswell JM, Yui MA, Burt DW, Kelley VE: Increased tumor necrosis factor and IL-1β gene expression in the kidneys of mice with lupus nephritis. J Immunol 1988, 141:3050–3054
14. Diamond JR, Pesek I: Glomerular tumor necrosis factor and interleukin 1 during acute aminonucleoside nephrosis: an immunohistochemical study. Lab Invest 1991, 64:21–28
15. Tipping PG, Leong TW, Holdsworth SR: Tumor necrosis factor production by glomerular macrophages in anti-glomerular basement membrane glomerulonephritis in rabbits. Lab Invest 1991, 65:272–279
16. Pennica D, Nedwin GE, Hayflick JS, Seeburg PH, Derynck R, Palladino MA, Kohr WJ, Aggarwal BB, Goeddel DV: Human tumour necrosis factor: precursor structure, expression and homology to lymphotoxin. Nature 1984, 312:724–729
17. Crabb ID, Hughes SS, Hicks DG, Puzas JE, Tsao GJY, Rosier RN: Nonradioactive *in situ* hybridization using digoxigenin-labeled oligonucleotides. Am J Pathol 1992, 141:579–589
18. Pringle JH, Primrose L, Kind CN, Talbot IC, Lauder I: *In situ* hybridization demonstration of poly-adenylated RNA sequences in formalin-fixed paraffin sections using a biotinylated oligonucleotide poly d(T) probe. J Pathol 1989, 158:279–286
19. Boyce NW, Fernando NS, Neale TJ, Holdsworth SR: Acute pulmonary and renal injury after administration of heterologous anti-lung antibodies in the rat: characterization of ultrastructural binding sites, basement membrane epitopes, and inflammatory mediation systems. Lab Invest 1991, 64:272–278
20. Fernando NS, Boyce NW, Holdsworth SR, Neale TJ: Localization of antiglomerular basement membrane-binding polyclonal antibodies in rat lung and kidney using post-embedding immunogold electron microscopy. Br J Exp Pathol 1989, 70:557–565
21. McIntosh JEA, Murray-McIntosh RP: MacRIA version 1.01. Wellington School of Medicine, Wellington, New Zealand, 1990
22. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970, 227:680–685
23. Ehrenreich T, Churg J: Pathology of membranous nephropathy. Pathol Annu 1968, 3:145–186

24. Suranyi MG, Guasch A, Hall BM, Myers BD: Elevated levels of tumor necrosis factorα in the nephrotic syndrome in humans. Am J Kidney Dis 1993, 21:251–259
25. Jevnikar AM, Wuthrich RP, Brennan DC, Maslinski W, Glimcher LH, Rubin-Kelley VE: TNF-α is expressed on the surface of kidney proximal tubular cells. Tranplant Proc 1991, 23:231–232
26. Yard BA, Daha MR, Kooyman S, Couthino M, Bruijn JA, Paape ME, Schrama E, van Es LA, van der Woude FJ: IL-1α stimulated TNF-α production by cultured human proximal tubular epithelial cells. Kidney Int 1992, 42:383–389
27. Matsumoto K: Increased release of tumor necrosis factor-α by monocytes from patients with glomerulonephritis. Clin Nephrol 1993, 40:148–154
28. Kerjaschki D: Molecular pathogenesis of membranous nephropathy. Kidney Int 1992, 41:1090–1105
29. Barabas AZ, Lannigan R: Induction of an autologous immune complex glomerulonephritis in the rat by intravenous injection of heterologous anti-rat kidney antibody. I. Production of chronic progressive immune complex glomerulonephritis. Br J Exp Pathol 1974, 55: 47–55
30. Heymann W, Knutec EP, Wilson SGF, Hunter JLP, Hackel DB, Okuda R, Cuppage L: Experimental autoimmune renal disease in rats. Ann NY Acad Sci 1965, 124:310–326
31. Neale TJ, Wilson CB: Glomerular antigens in Heymann's nephritis: reactivity of eluted and circulating antibody. J Immunol 1982, 128:323–330
32. Neale TJ, Woodroffe AJ, Wilson CB: Spontaneous glomerulonephritis in rabbits: the role of a glomerular capillary antigen. Kidney Int 1984, 26:701–711
33. Neale TJ, Ullrich R, Ojha P, Poczewski H, Verhoeven AJ, Kerjaschki D: Reactive oxygen species and neutrophil respiratory burst cytochrome b558 are produced by kidney glomerular cells in passive Heymann nephritis. Proc Natl Acad Sci USA 1993, 90:3645–3649
34. Schonermark M, Deppisch R, Riedasch G, Rother K, Hansch GM: Induction of mediator release from human glomerular mesangial cells by the terminal complement components C5b-9. Int Arch Allerg Appl Immunol 1991, 96:331–337
35. Neale TJ, Ojha PP, Exner M, Poczewski H, Rüger BM, Witztum JL, Davis P, Kerjaschki D: Proteinuria in passive Heymann nephritis is associated with lipid peroxidation and formation of adducts on type IV collagen. J Clin Invest 1994, 94:1577–1584
36. Sacks SH, Zhou W, Pani A, Campbell RD, Martin J: Complement C3 gene expression and regulation in human glomerular epithelial cells. Immunology 1993, 79:348–354
37. Bertani T, Abbate M, Zoja C, Corna D, Perico N, Ghezzi P, Remuzzi G: Tumor necrosis factor induces glomerular damage in the rabbit. Am J Pathol 1989, 134:419–430
38. Hruby ZW, Shirota K, Jothy S, Lowry RP: Antiserum against tumor necrosis factor-α and a protease inhibitor reduce immune glomerular injury. Kidney Int 1991, 40:43–51
39. Tomosugi NI, Cashman SJ, Hay H, Pusey CD, Evans DJ, Shaw A, Rees AJ: Modulation of antibody-mediated glomerular injury by bacterial lipopolysaccharide, tumor necrosis factor and IL-1. J Immunol 1989, 142:3470–3090
40. Nakamura T, Ebihara I, Fukui M, Takahashi T, Tomino Y, Koide H: Altered glomerular steady-state levels of tumour necrosis factor-α mRNA during nephrotic and sclerotic phases of puromycin aminonucleoside nephrosis in rats. Clin Sci 1993, 84:349–356
41. Brennan DC, Yui MA, Wuthrich RP, Kelley VE: Tumor necrosis factor and IL-1 in New Zealand Black/White mice: enhanced gene expression and acceleration of renal injury. J Immunol 1989, 143:3470–3475
42. Jacob CO, McDevitt HO: Tumor necrosis factor-α in murine autoimmune lupus nephritis. Nature 1988, 331: 354–357
43. Hruby ZW, Cybulsky AV, Lowry RP: Effects of tumor necrosis factor on glomerular mesangial and epithelial cells in culture. Nephron 1990, 56:410–413
44. Johnson R, Yamabe H, Chen YP, Campbell C, Gordon K, Baker P, Lovett D, Couser WG: Glomerular epithelial cells secrete a glomerular basement membrane-degrading metalloproteinase. J Am Soc Nephrol 1992, 2:1388–1397
45. Hanemaaijer R, Koolwijk P, le Clercq L, de Vree WJ, van Hinsbergh VW: Regulation of matrix metalloproteinase expression in human vein and microvascular endothelial cells: effects of tumour necrosis factor α, interleukin 1 and phorbol ester. Biochem J 1993, 296: 803–809
46. Radeke HH, Meier B, Topley N, Flöege J, Habermehl GG, Resch K: Interleukin-1α and tumor necrosis factor-α induce oxygen radical production in mesangial cells. Kidney Int 1990, 37:767–775
47. Satriano JA, Shuldiner M, Hora K, Xing Y, Shan Z, Schlondorff D: Oxygen radicals as second messengers for expression of the monocyte chemoattractant protein, JE/MCP-1, and the monocyte colony-stimulating factor, CSF-1, in response to tumor necrosis factor-α and immunoglobulin G: evidence for involvement of reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent oxidase. J Clin Invest 1993, 92:1564–1571