Virchows Archiv (1994) 424:459–464

ORIGINAL ARTICLE

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Cellular localization of inflammatory cytokines in human glomerulonephritis

Received: 28 October 1993 / Accepted: 18 March 1994

Abstract We evaluated the expression of inflammatory cytokines in renal tissues obtained from 45 patients with several types of glomerulonephritis. Immunofluorescence studies with specific antibodies to interleukin (IL)-1α, IL-1β, IL-6, tumour necrosis factor (TNF)-α, and TNF-β showed intense cytoplasmic staining in the glomeruli and interstitium. Cells positive for these cytokines were found frequently in tissue from patients with lupus nephritis (WHO Class IV) and membranoproliferative glomerulonephritis, and, to a lesser extent, in tissue from patients with mesangial proliferative glomerulonephritis, Henoch-Schönlein purpura nephritis, and minimal change nephrotic syndrome. Most of these cells were dual-stained with a monoclonal antibody to monocytes-macrophages. In situ hybridization for cytokine mRNA, combined with immunoperoxidase staining for monocytes-macrophages, detected IL-1α, IL-6, and TNF-α mRNA in monocytes-macrophages infiltrating the glomeruli and interstitium. Occasionally, there was weak or moderate immunostaining for IL-1α, IL-6, and TNF-α in the glomerular mesangial and epithelial cells, but in situ hybridization signals were rarely found in these loci. These findings suggest that infiltrating monocytes-macrophages, rather than resident glomerular cells, are the major source of inflammatory cytokines in human glomerulonephritis.

Key words Interleukin · Tumour necrosis factor
Glomerulonephritis · Monocytes · Macrophages

Introduction

Recent clinical and experimental studies indicate that various cytokines are involved in glomerular injury [2, 5, 14]. The augmented expression of interleukin (IL)-1 at mRNA and protein levels has been observed in glomeruli in mice with lupus nephritis [3, 4], in rabbits with nephrotoxic serum nephritis [18], and in rats with focal glomerular sclerosis [6] or immune complex nephritis [20]. The participation of tumour necrosis factor (TNF)-α has been documented in lupus mice [4], rabbits with anti-glomerular basement membrane (GBM) disease [17], and rats with acute aminonucleoside nephrosis [6]. In human mesangial proliferative glomerulonephritis, the enhanced expression of IL-6 in glomeruli and its appearance in the urine have been reported [11]. These cytokines can be produced either by monocytes-macrophages lineage or by endothelial and vascular smooth muscle cells [8]. In addition, as indicated by culture studies, mesangial cells may carry out the local synthesis of IL-1 [14], IL-6 [11], and TNF-α [2].

We recently reported the concomitant expression of several cytokines in the glomeruli of patients with IgA nephritis [24]. To extend our previous studies and to further elucidate the cellular origin of pro-inflammatory cytokines in glomeruli, we analysed IL and TNF expression in kidney specimens biopsied from patients with several types of glomerular diseases.

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Materials and methods

The kidney tissues were obtained by percutaneous needle biopsy from 45 patients with glomerular disease: 11 patients had minimal change nephrotic syndrome, 11 had Henoch-Schönlein purpura nephritis, 10 had mesangial proliferative (IgA-negative) glomerulonephritis, 8 had lupus nephritis (WHO Class II; 3 and Class IV; 5), and 5 had membranoproliferative glomerulonephritis. Histologically normal portions of 3 other kidney tissues, obtained from patients with renal tumours or renal trauma served as normal controls. For light microscopic observation, the tissues were fixed in 10% buffered formalin and embedded in paraffin. Specimens for immunofluorescence were snap-frozen in dry ice and acetone, and those for immunoperoxidase staining and in situ hybridization were fixed in paraformaldehyde-lysine-periodate (PLP) fixative. The tissues were then embedded in OCT compound, and stored at −70 °C until use.

Indirect immunofluorescent staining was performed as described previously [22, 24]. In brief, the sections were incubated with the primary antibodies, washed in phosphate-buffered saline (PBS), and then reacted with the appropriate secondary antibodies. Cell nuclei were counterstained with ethidium bromide. As the primary antibodies we used; rabbit antibody to human IL-1α (Genzyme, Cambridge, Mass., USA), rabbit antibody to human IL-1β (Genzyme), mouse monoclonal antibody to human IL-6

(Collaborative Bio, Bedford, Mass., USA), rabbit antibody to human IL-6 (Genzyme), mouse monoclonal antibody to human TNF-α (Hayashibara, Okayama, Japan), rabbit antibody to human TNF-α (Hayashibara), rabbit antibody to human TNF-β (Hayashibara), mouse monoclonal antibody to human leukocyte common antigen (CD45; DAKO, Glostrup, Denmark), mouse monoclonal antibody to monocyte-macrophages (CD14) (anti-Leu-M3; Becton-Dickinson, Bedford, Mass., USA), mouse monoclonal antibody to human macrophages (CD68) (DAKO-CD68, DAKO), mouse monoclonal antibody to T lymphocytes (T1B; Coulter, Hialeah, Fla., USA), and mouse monoclonal antibody to B lymphocytes (anti-Leu12; Becton-Dickinson). As the secondary antibodies we used; fluorescein- or peroxidase-conjugated F(ab')2 goat anti-rabbit IgG (Cappel, Durham, N.C., USA) and fluorescein- or rhodamine conjugated goat anti-mouse IgG (Cappel). The secondary antibodies were preabsorbed with normal human plasma to avoid non-specific binding to human tissues.

Dual-fluorochrome labelling was performed as follows [15]: monocytes-macrophages, T lymphocytes, or B lymphocytes were stained with rhodamine-conjugated goat anti-mouse IgG, and IL and TNF were detected with fluorescein-conjugated goat anti-rabbit IgG.

Immunoperoxidase staining was performed as reported previously [15, 24]. In brief, PLP-fixed tissue was cut into thin sections, and treated with periodate and sodium borohydroxide to inhibit endogenous peroxidase. Each section was reacted with anti-monocyte-marcophage antibody, then with the appropriate secondary antibody, and finally with the enzyme substrate.

In each section, which contained at least three glomeruli, we counted all positive infiltrating cells within the glomeruli and in the interstitium, the number of glomeruli present, and the total numbef of interstitial (excluding tubular epithelial cells and arterioles) cells, as described previously. The population of cytokine-positive cells in the glomeruli and interstitium was expressed as the number of reactive cells per glomerular crosssection and the number of reactive cells per 100 interstitial cells, respectively.

In situ hybridization was performed in combination with immunocytochemistry on the same section, with a slight modification [24] of the method originally described by Terenghi and Polak [16]. Sections were first stained with immunoperoxidase for monocytes-macrophages (CD14), and were then hybridized with cytokine oligonucleotide probes. The in situ hybridization procedure was based on the protocol described elsewhere [23, 24]. The hybridization probes for IL-1α, IL-6, and TNF-α were purchased from British Biotech (Oxford, UK). Each probe, a mixture composed of synthetic single-stranded, antisense oligonucleotides specific for the exons of the respective cytokine, was labeled with ^{35}S-dATP (NEN, Boston, Mass., USA) using terminal deoxynucleotide transferase to a specific radioactivity of 1×10^{8}–10^{9} dpm/µg.

The thin section of formalin-fixed tissue was mounted on a slide, then washed with SSC (standard saline citrate), as described previously [23, 24]. Prehybridization was performed with Denhardt's solution. The section was then dehydrated with a graded ethanol, treated with chloroform, and immersed in 100% ethanol. The hybridization mixture (100 µl), containing the ^{35}S-labelled probe (10^{5} dpm/section) in hybridization buffer, was applied to each section. After incubation at 42 °C overnight, the slide was washed sequentially in ×4 –×1 SSC, and dehydrated with an ethanol series (60–100%). Autoradiography was performed by dipping the slide into Ilford K5 emulsion diluted 1:1 with distilled water; the slide was then kept in a black box at 4 °C for 3–4 weeks. The autoradiogram was developed in Kodak developer, fixed, and counterstained with haematoxylin-eosin.

Controls consisted of in situ hybridization of probes with peripheral blood mononuclear cells stimulated with lipopolysaccharide, which cells are known to express IL-1 and TNF-α mRNA. Negative controls were carried out by hybridizing sections with a synthetic oligonucleotide (RANDOMER, 36 base, NEN) labelled with ^{35}S, or by using sections pretreated with RNase [23].

Results

No IL or TNF immunofluorescent staining was observed in control kidneys. In tissues from patients with glome-

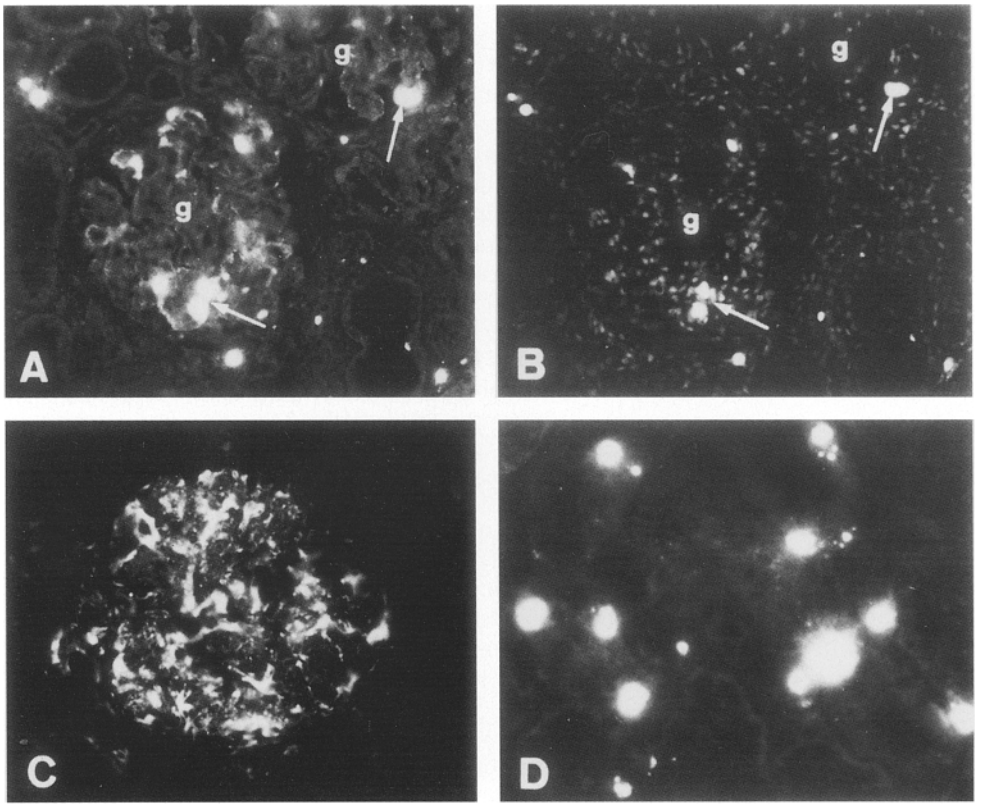

Fig. 1 IL-1α and IL-1β immunoreactivity in kidney tissues from patients with lupus nephritis (WHO Class IV). **A, B** Dual staining with polyclonal antibody to IL-α (**A**) and monoclonal antibody to monocytes-macrophages (**B**) in the same section. Note cytoplasmic staining *(arrows)* for IL-1α and labelling of the same cells for monocytes-macrophages. Weakly positive IL-1α staining is also seen in resident glomerular cells (**A**). Cellular nuclei stained by ethidium bromide are weakly visible (**B**). **C** Staining for IL-1α in resident glomerular cells. **D** Intense cytoplasmic staining for IL-1β in cells infiltrating the interstitium, *g* glomerulus. Original magnification; **A, B,** ×200; **C, D,** ×400

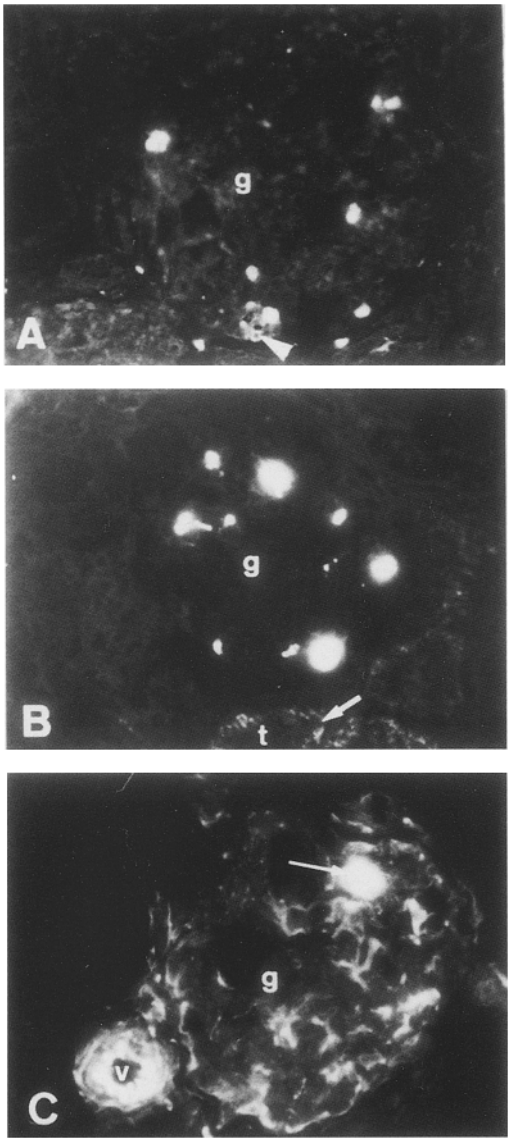

Fig. 2 Immunofluorescent staining of IL-6 and TNF-α. **A** IL-6 staining in lupus nephritis (WHO Class IV). Round, cytoplasmic staining in infiltrating cells and patchy staining in resident glomerular cells *(arrowhead)*. **B** IL-6 staining in membranoproliferative glomerulonephritis. Cells infiltrating a glomerulus are intensely positive. Tubular epithelial cells are weakly labelled *(arrow)*. **C** TNF-α staining in Henoch-Schönlein purpura nephritis. TNF-α positivity is intense in an infiltrating cell *(arrow)* and blood vessel (*v*), and moderate in resident glomerular cells, *g* glomerulus; *t* tubular epithelium; *v* blood vessel. Original magnification, A–C ×400

rulonephritis, cytokine-positive cells were detected in the glomeruli and interstitium (Figs. 1, 2). The positive cells showed bright, round-shaped immunofluorescence. Dual staining or staining of serial sections was performed using a monoclonal antibody to human leukocyte common antigen (CD45) or anti-monocyte-macrophage (CD14 and CD68) antibody and anti-cytokine antibody, which revealed that most of the cytokine-positive cells were monocytes-macrophages. The findings obtained with anti-CD14 antibody and anti-CD68 antibody were

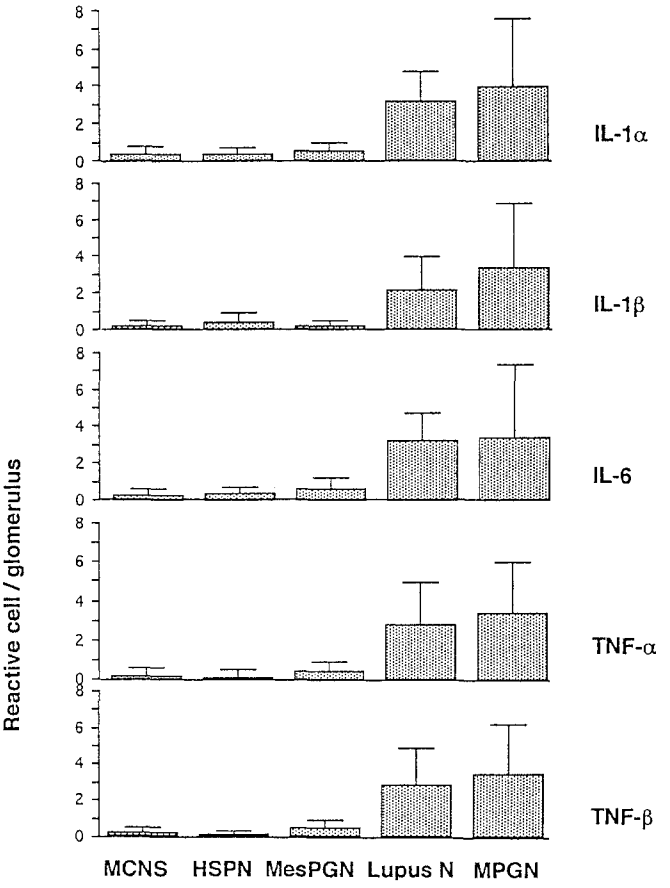

Fig. 3 Proportions of glomerular cells reactive by immunofluorescence with antibodies to IL-1α, IL-1β, IL-6, TNF-α, and TNF-β. *MCNS* minimal change nephrotic syndrome; *HSPN* Henoch-Schönlein purpura nephritis; *MesPGN* mesangial proliferative (IgA-negative) glomerulonephritis; *N* nephritis; *MPGN* membranoproliferative glomerulonephritis

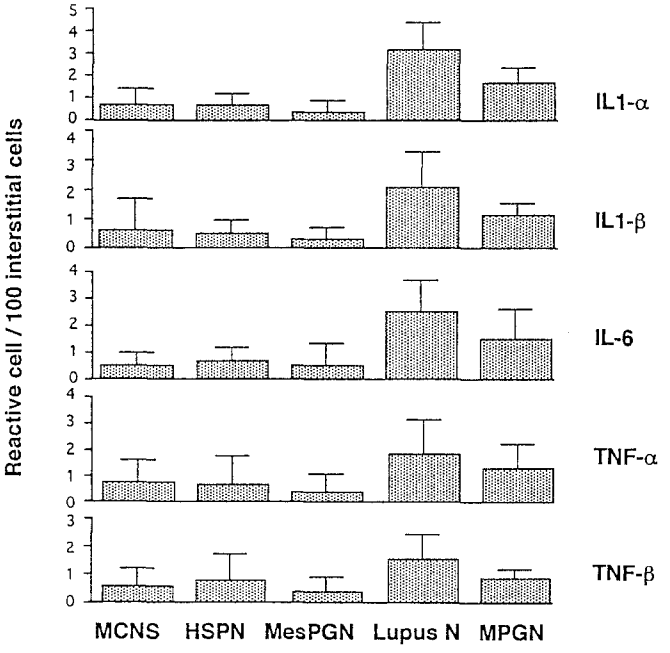

Fig. 4 Population of cells immunoreactive for cytokines in the renal interstitium. Abbreviations (see Fig. 3)

Table 1 Glomerular resident cell staining for cytokines in glomerular diseases (Staining intensity was graded as weak (1+) and moderate (2+). Localization of cytokines is shown as: *Epi* glomerular visceral epithelium; *Mes* mesangium; *End* glomerular endothelium)

	No. of patients examined	No. of patients having positive immunofluorescence				
		IL-1α	IL-1β	IL-6	TNF-α	TNF-β
Localization		Epi, Mes	Epi, Mes	Mes	Epi, End, Mes	Epi
Disease						
Minimal change NS	11	0	0	0	0	0
Henoch-Schönlein purpura nephritis	11	0	0	2(1+)	5(1+) 1(2+)	0
Mesangial proliferative GN	10	2(1+)	0	1(1+)	3(1+) 2(2+)	0
Lupus nephritis						
WHO Class II3	3	1(1+)	0	2(1+)	3(1+)	0
WHO Class IV	5	2(1+) 2(2+)	3(1+)	4(1+)	2(1+) 3(2+)	1(1+)
Membranoproliferative GN	5	2(1+)	2(1+)	2(1+)	4(1+) 1(2+)	0
Normal	4	0	0	0	0	0

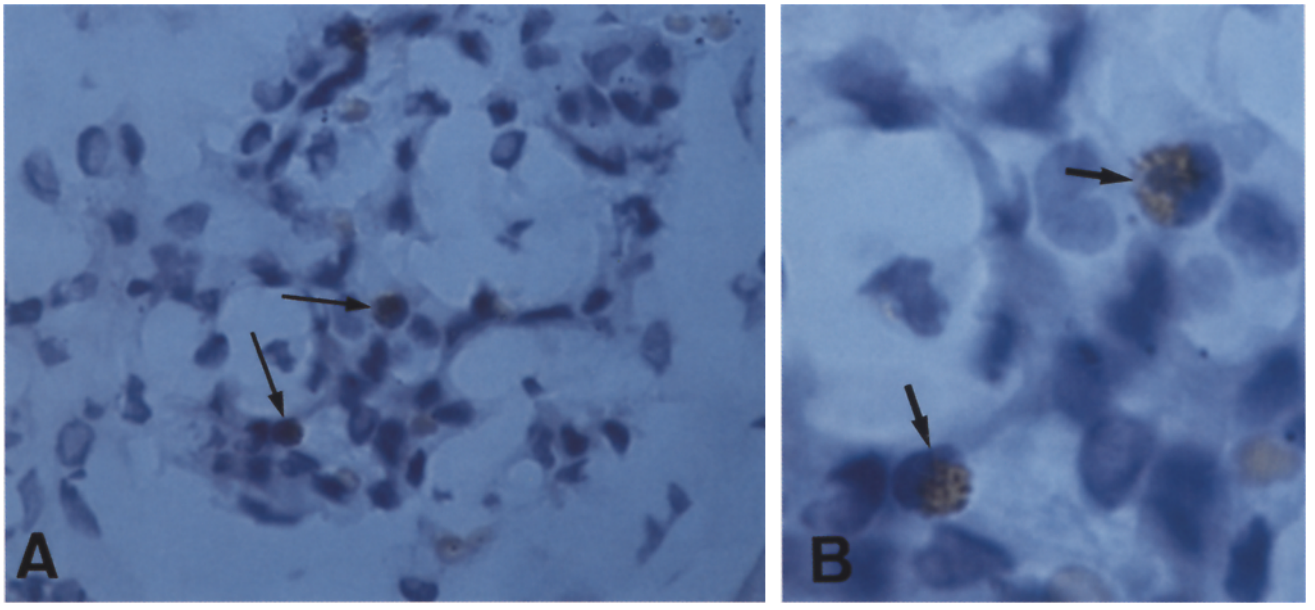

Fig. 5 Intraglomerular monocytes-macrophages expressing IL-1α mRNA, identified by double labelling by immunocytochemistry and in situ hybridization. Immunoperoxidase staining (brown in cytoplasm) with anti-monocyte-macrophage (CD14) antibody and in situ hybridization (dark grains in cytoplasm), using a ^{35}S-labelled IL-1α antisense oligonucleotide probe, were performed sequentially on the same tissue section from a patient with mesangial proliferative glomerulonephritis. Positive cells are indicated by *arrows*. **A,** × 800; **B** (higher magnification of positive cells), ×1,000

similar. The population of cytokine-positive cells in the glomeruli was high in diffuse proliferative lupus nephritis and membranoproliferative glomerulonephritis (Fig. 3). The percentage of positive cells in the interstitium was also high in these disease (Fig. 4).

In situ hybridization, using oligonucleotide probes for IL-1α, IL-6, and TNF-α, in combination with immunoperoxidase staining for monocytes-macrophages, was performed on tissues from the six patients whose specimens showed intense immunofluorescence for these cytokines. Clusters of silver grains, corresponding to IL-1α, IL-6, and TNF-α transcripts, were observed in the infiltrating monocytes-macrophages, which were brown on peroxidase staining (Fig. 5). Sections hybridized with a control probe or tissue treated with RNase prior to hybridization showed no significant grain counts.

In addition to the staining of the infiltrating cells, there was some resident glomerular cell staining. The distribution and frequency of cytokine staining are summarized in Table 1. Labelling for TNF-α was more frequent

and more diffuse than that for IL-1α, IL-1β, IL-6, and TNF-β. TNF-α was localized predominantly in the glomerular visceral epithelial cells, and, to a lesser extent, in the glomerular mesangial and endothelial cells. In some patients, glomerular cells were labelled for IL-1α, IL-1β, IL-6, and TNF-β. These stainings were generally weak, except for IL-1α in two patients with diffuse proliferative lupus nephritis (Fig. 1C). In the in situ hybridization study, positive signals for IL-1α, IL-6, and TNF-α were rarely observed in resident glomerular cells.

Immunofluorescence for IL-1α and IL-1β was frequently positive in the media of small blood vessels. IL-6 was occasionally stained in the cytoplasm of tubular epithelial cells. TNF-α staining was frequently observed in the epithelial cells of the Bowman's capsule, in the intima and media of small blood vessels, and in the interstitial cells.

Discussion

The immunocytochemistry and in situ hybridization techniques used here demonstrated the local expression of IL-1, IL-6, and TNF in the kidneys of patients with several types of glomerulonephritis. The majority of cells immunoreactive for IL-1, IL-6, TNF-α, and TNF-β were monocytes-macrophages infiltrating the glomeruli and interstitium. The production of IL and TNF by mesangial cells from normal glomeruli in vitro has been well documented [2, 11, 14]. Thus, we had anticipated that substantial amounts of cytokines were synthesized by glomerular mesangial cells. However, our immunostaining and in situ hybridization findings suggested that monocytes-macrophages are the major source of pro-inflammatory cytokines. The staining intensity in the immunofluorescence study and the signal density in the in situ hybridization study were prominent in infiltrating monocytes-macrophages. Thus, it appears that cytokine production by monocytes-macrophages is more pronounced than that by resident glomerular cells. The contribution of monocytes-macrophages, rather than mesangial cells, to local IL-1 and TNF synthesis has been emphasized in studies of animal models. Tipping and co-workers [17, 18] reported that, in experimental anti-GBM nephritis, where injury was macrophage-dependent, IL-1 and TNF production was macrophage-dependent, and infiltrating glomerular macrophages were the major source of IL-1 and TNF. In acute aminonucleoside nephrosis in rats, Diamond and Pesek [6] demonstrated the correlation in number between glomerular macrophages and TNF- or IL-1-positive glomerular cells immunohistochemically. Further, recent human studies by Waldherr and co-workers [13, 19], using immunohistochemistry and in situ hybridization, have shown enhanced expression of IL-1β, TNF-α, interferon and IL-2 receptors in infiltrating monocytes-macrophages in tissues from patients with anti-neutrophil cytoplasmic autoantibody-positive glomerulonephritis and mesangial IgA nephritis. Our own studies in IgA nephritis [24] have also demonstrated enhanced expression of IL-1, IL-6, and TNF-α at mRNA and protein levels in infiltrating monocytes-macrophages.

The use of tissue culture and histochemical and monoclonal antibody techniques has established that monocytes-macrophages infiltrate glomeruli and glomerular crescents in rapidly progressive glomerulonephritis, membranoproliferative glomerulonephritis, lupus nephritis, and IgA nephritis in man [1, 9, 22]. Here, we found that the tissues of patients with diffuse lupus nephritis and membranoproliferative glomerulonephritis showed numerous monocytes-macrophages infiltrating the glomeruli and interstitium; these cell expressed IL-1, IL-6, and TNF simultaneously. Although we found this cell type in other forms of glomerular disease, including minimal change nephrotic syndrome, their population was lower than in these two diseases. In addition to their well-known functions of procoagulant activity, phagocytosis, oxygen radical generation, and release of GBM-degrading proteinases [12, 21], monocytes-macrophages may be important as a cellular source of these pro-inflammatory cytokines that potentially promote the proliferation of glomerular mesangial cells. A recent study by Diamond et al. [7] using an experimental model of nephrotic syndrome, has indicated that infiltrating glomerular macrophages, by augmenting mesangial cell proliferation, may be important effectors in the progression of initial glomerular injury to glomerular cell proliferation and further glomerulosclerosis.

The mesangial staining for cytokines shown here is consistent with some and inconsistent with other previous reports from other laboratories. Fukatsu et al. [10] have shown rather broad distribution of immunoreactive IL-6. They found mesangil IL-6 was found in mesangial proliferative glomerulonephritis and in non-proliferative glomerulonephritis. They also detected it in the epithelium of the Bowman's capsule, synechiae, tubular epithelial cells, and arterial muscle cells, but not in infiltrating leukocytes. Waldherr et al. [19] have briefly described IL-6 expression in glomeruli with marked mesangial proliferation and/or expansion, and have also shown this expression in interstitial and tubular epithelial cells of tissues with widespread tubulo-interstitial damage. We observed only weak IL-6 immunoreactivity in resident mesangial cells of glomeruli showing marked increases in mesangial cells and matrix, and this weak immunoreactivity was also seen in some cells in the renal tubular epithelium. These discrepancies could be due to differences in the specimens analysed and antibodies used.

In conclusion, these results indicate the concomitant expression of several pro-inflammatory cytokines in infiltrating monocytes-macrophages, and, to a lesser extent, in the resident renal cells of glomerulonephritis. The multifunctional properties of these factors may play a synergistic role in the inflammatory process in glomeruli.

Acknowledgements This work was supported by a Grant for "Progressive Renal Diseases" from the Ministry of Health and Welfare project for "Specially selected diseases" (K.Y.), a Grant from the Osaka Kidney Foundation (OKF94-0001, T.T.), and A Grant-in-aid for Scientific Research (05770577) from the Ministry of Education (K.M.). The authors thanks Ms. Y. Shima for preparing the manuscript.

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