Circulating Factors in Sera or Peripheral Blood Mononuclear Cells in Patients with Membranous Nephropathy or Diabetic Nephropathy

In order to investigate the status of some circulating factors in nephrotic syndrome, we examined the secretion of monocyte chemotactic peptide (MCP)-1, tumor necrosis factor (TNF) α or fibronectin in sera or by peripheral blood mononuclear cells (PBMC) from patients with membranous nephropathy (MN), diabetic nephropathy (DN) or minimal change disease (MCD). Also the effects of PBMC or sera on human mesangial cells (MC) were evaluated. Serum TNF α levels were higher in patients with MN than in controls, but PBMC exhibited no differences in TNF α production between patients and controls. Serum fibronectin levels were higher in patients with MN than in controls. PBMC from diabetic patients with or without nephropathy produced more MCP-1 than cells from controls. When MC were cultured with PBMC supernatants from patients, TNF α levels in PBMC supernatants correlated with production of MCP-1 or fibronectin by MC. PBMC supernatants obtained from patients with MCD and MN decreased MCP-1 production by MC, but did not affect thymidine incorporation or fibronectin production by MC. Sera obtained from patients with DN and MCD reduced thymidine incorporation in MC. In summary, serum TNF α or fibronectin levels were increased in patients with MN that is known to progress to renal failure. MCP-1 Production was increased by PBMC obtained from diabetic patients with or without nephropathy. Also TNF α production by PBMC in individual patients may affect the pathophysiology of their MC.
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Key Words : *Monocyte chemoattractant protein-1; Tumor necrosis factor; Fibronectins; Nephrotic syndrome; Diabetic nephropathies*

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INTRODUCTION

The pathogenetic mechanisms of nephrotic syndrome remain still undefined. Serum factors or lymphokines such as vascular permeability factors or soluble immune response suppressor have been suggested to play an important role in nephrotic syndrome such as minimal change disease (MCD) (1-3). Peripheral blood mononuclear cells (PBMC) obtained from patients with glomerular diseases such as IgA nephropathy can produce several mediators including cytokines (4-6). It is known that circulating molecules and filtration residues constantly perfuse and affect the mesangium which has a high capacity to process them (7). Therefore, it is conceivable that PBMC or sera can interact with mesangial cells (MC) in nephrotic syndrome. Monocytes can affect cellularity and production of extracellular matrix (ECM) protein and cytokines in MC, which may be related to the pathogenesis or progression of glomerular diseases (8-13).

The chemokine superfamily is important in leukocyte chemotaxis and activation. Monocyte chemotactic peptide-1 (MCP-1) is a Cysteine-Cysteine (C-C) chemokine for monocytes (14-16). This is expressed in many cells, including MC and monocytes, and is responsible for the bulk of the monocyte chemotactic activity produced by cultured human MC. Increased glomerular expression has been shown in experimental glomerular diseases (17-19), and urinary levels of MCP-1 were increased also in patients with membranous nephropathy (MN) and diabetic nephropathy (DN) (20). Tumor necrosis factor (TNF) α plays a prominent role in glomerular diseases. It induces proteinuria and affects the production of proinflammatory cytokines or ECM protein (21-23). Especially, it is known to be one of the potent inducers of MCP-1 (14). On the other hand, plasma fibronectin concentration was reported to be increased in diabetic patients with microalbuminuria (24).

Therefore, in order to investigate the status of some circulating factors, we examined the concentrations of

MCP-1, TNF α, or fibronectin in sera or PBMC supernatants obtained from patients with membranous nephropathy or diabetic nephropathy. Also the effects of PBMC or sera on human MC were evaluated by examining the thymidine incorporation and the production of fibronectin and MCP-1 in MC.

MATERIALS AND METHODS

Patients

We evaluated fifteen patients with nephrotic syndrome, including MN, MCD, and DN, as well as five healthy volunteers. Also five diabetic patients without proteinuria were included as a disease control group of DN. All diabetic patients had non-insulin dependent diabetes mellitus. The clinical profiles of patients are summarized in Table 1.

Mononuclear cell culture

Heparinized blood was obtained from patients. PBMC were prepared by Ficoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden) density gradient centrifugation and were plated in RPMI media with 10% fetal calf serum (Sigma Chemical Co., St. Louis, USA) as previously described (16). They were treated with concanavalin A (Sigma Chemical Co., St. Louis, USA) at 20 μg/ml for 72 hrs. After that the supernatant was collected by centrifugation, filtered and stored at -70℃.

Human MC culture

Portions of normal renal cortex were obtained from human kidneys immediately after surgical nephrectomy. Collagenase (Gibco BRL, Grand Island, USA) treated glomerular cores were plated on culture dishes in DMEM media (Gibco BRL) containing 17% heat-inactivated fetal calf serum (25, 26). Cells in culture displayed the typical spindle or stellate morphology. Immunofluorescent staining of the cells was negative with antibodies to common leukocyte antigen and factor VIII, and the cells were capable of growth in D-valine substituted medium. Near confluent MC in the third to fifth passage were transferred to a 24-well plate, and were rested in serum-free medium for 48 hrs. MC then were cultured for 24 hrs after adding 50% PBMC supernatant or 50% sera obtained from patients. The media were exchanged with media without fetal calf serum that contained 2% bovine serum albumin. Then MC supernatants were collected for measurements of MCP-1 after 24 hrs and fibronectin after 72 hrs.

Measurements of MCP-1 and TNF α concentrations

Quantitative determination of MCP-1 and TNF α concentrations in sera and culture supernatants of PBMC and MC was performed using a human enzyme-linked immunosorbent assay (ELISA) kit (Quantikine; R & D, Minneapolis, USA). This is a multiple-sandwich solid-phase enzyme linked immunoassay, which uses monoclonal antibodies raised against human MCP-1 and TNF α (20). Standards and diluted samples were pipetted into the wells. After washing away unbound substances, an enzyme-linked polyclonal antibody specific for MCP-1 and TNF α was added to the wells. Following a wash, a substrate solution was added to the wells and the intensity of the color was measured. The sensitivity of the ELISA for MCP-1 was 5 pg/ml and that for TNF α was 4.4 pg/ml. Results are expressed as the mean of duplicate determinations.

Measurements of fibronectin concentration

Quantitative determination of fibronectin concentration in sera and MC supernatants was performed using a human ELISA kit (Quantikine; R & D, Minneapolis, USA). This is a quantitative competitive binding enzyme immunoassay, which uses polyclonal antibodies specific for fibronectin. Fibronectin in standards, diluted samples and controls compete with conjugate to bind the antibody. The sensitivity of the assay is 0.42 μg/ml. Results are expressed as the mean of duplicate determinations.

Table 1. Patients subjected to this study

Category	Number	Age (year)	Proteinuria (g/day)	Serum creatinine (mg/dl)	Serum cholesterol (mg/dl)	Serum albumin (g/dl)
Control	5	35±11	–	0.8±0.2	189± 32	3.9±0.3
Minimal change disease	5	25± 4	21±14	0.9±0.2	498±177	1.9±0.6
Membranous nephropathy	5	51± 6	12± 6	0.9±0.1	265± 49	2.7±0.7
Diabetic nephropathy	5	55± 9	10± 6	1.6±0.3	232± 32	2.7±1.3
Diabetes without nephropathy	5	59±14	–	0.8±0.2	144± 44	3.8±0.4

Data are shown as Mean ± S.D.

Thymidine incorporation assays

MC were transferred to a 96-well plate, and were rested in serum-free medium for 48 hrs. MC were cultured in DMEM media without fetal calf serum for 8 hrs after adding 50% PBMC supernatant or sera obtained from patients. MC then were pulsed with one μCi per well of [^{3}H] thymidine (Amersham, Buckinghamshire, England) (26). After 16 hrs the monolayers were washed with PBS, precipitated, and washed twice with 10% TCA. After solubilized in 1.0 N NaOH, the contents of each well were neutralized with HCl and counted in a liquid scintillation counter. Results are expressed as the mean of triplicate determinations.

Statistics

Results are expressed as the mean±S.D. of the measurements. Comparisons among groups were made by analysis of variance. Comparisons between groups were made using the Student's unpaired t-test. Significance was assigned at the $p<0.05$ levels.

RESULTS

TNF α concentration in sera and PBMC supernatants from patients

The serum TNF α levels were higher in patients with MN ($p<0.05$), not in those with MCD or DN, than in controls (Table 2, Fig. 1). PBMC, when stimulated with concanavalin A, exhibited no differences in TNF α production between patients and controls.

MCP-1 production by PBMC

A composite standard curve from 7 separate ELISAs showed the reproducibility of the MCP-1 assay. When stimulated with concanavalin A, PBMC from diabetic patients with (166±15 ng/ml) or without nephropathy (168±19 ng/ml) produced more MCP-1 than cells from controls (126±28 ng/ml) ($p<0.05$) (Table 3). But there was no relationship between MCP-1 and TNF α levels in PBMC supernatants from patients.

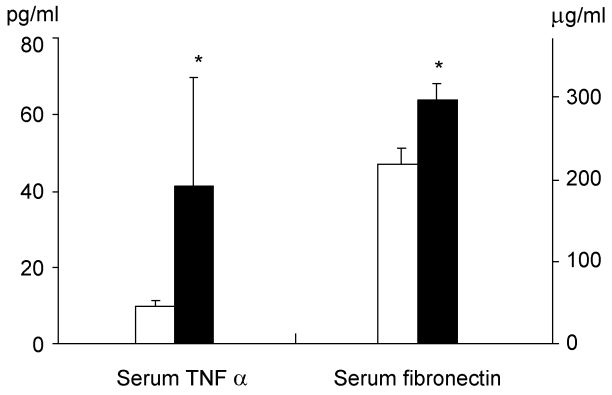

Fig. 1. The serum levels of TNF α and fibronectin in patients with MN. The serum levels of TNF α and fibronectin were higher in patients with MN (■) than in controls (□). * $p<0.05$ vs controls.

MCP-1 production by MC when incubated with PBMC supernatants

When MC were cultured with PBMC supernatants from patients, the relationship between TNF α in PBMC and MCP-1 production by MC was examined. The MCP-1 production by MC correlated with the TNF α levels in PBMC supernatants ($\gamma=0.42$, $p<0.05$) (Fig. 2). PBMC supernatants from patients with MCD or MN, except DN, decreased the MCP-1 production by MC after 24 hrs as compared to controls ($p<0.05$) (Table 3).

Fibronectin concentration in sera or culture supernatants of MC

The serum fibronectin levels were significantly higher in patients with MN (296±26 μg/ml), not in those with MCD or DN, than in controls (231±28 μg/ml) (p<

Table 2. TNF α concentrations

	TNF α (pg/ml)	
	Serum	PBMC supernatant
Control	9.7± 1.4	552±113
Minimal change disease	10.1± 1.0	500±112
Membranous nephropathy	41.2±30.0[a]	538±162
Diabetic nephropathy	14.6± 4.1	468±153
Diabetes without nephropathy	11.5± 1.9	486±151

[a] $P<0.05$ vs control

Table 3. MCP-1 production by PBMC from patients

	MCP-1 (ng/ml)	
	PBMC	PBMC treated mesangial cells
Control	126±28	7.2±0.9
Minimal change disease	155±59	5.5±0.8[a]
Membranous nephropathy	166±34	5.7±0.6[a]
Diabetic nephropathy	166±15[a]	6.1±0.8
Diabetes without nephropathy	168±19[a]	5.3±0.7[a]

[a] $P<0.05$ vs control

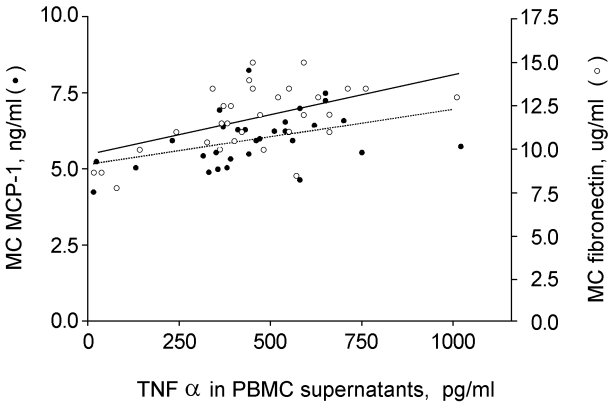

Fig. 2. Correlation between TNF α levels in PBMC and the production of MCP-1 or fibronectin by MC. When MC were cultured with PBMC supernatants from patients, the TNF α levels in PBMC supernatants correlated with the production of MCP-1 (γ =0.42, $p<0.05$)(·····) and fibronectin (γ =0.51, $p<0.005$) (——) by MC.

0.05) (Fig. 1). When MC were cultured with PBMC supernatants from patients, the fibronectin production by MC correlated with the TNF α levels in PBMC supernatants (γ =0.51, $p<0.005$) (Fig. 2). However, PBMC supernatants from patients in all groups did not affect the fibronectin production by MC after 3 days as compared to controls.

Thymidine incorporation by human MC

The thymidine incorporation by MC after treatment with sera was lower in patients with DN or MCD than in controls ($p<0.05$). [^{3}H] thymidine incorporation in MC was suppressed by culture supernatants of PBMC as compared to media alone (10,100±1,900 c.p.m.). Effects of PBMC supernatants on the thymidine incorporation in MC were not different between patients and controls (Table 4).

Table 4. Mesangial cell proliferation

	[^{3}H] Thymidine incorporation (c.p.m.)	
	Serum	PBMC supernatant
Control	15,200±1,500	4,100±1,300
Minimal change disease	10,900±1,900 [a]	4,400±1,500
Membranous nephropathy	10,800±3,100	4,500± 900
Diabetic nephropathy	8,800±3,400 [a]	4,200±1,200

[a] $P<0.05$ vs control

DISCUSSION

These experiments demonstrated some abnormalities in the secretion of MCP-1, TNF α, or fibronectin in sera or by PBMC obtained from patients with nephrotic syndrome.

The levels of serum TNF α were increased in patients with MN that is known to progress to renal failure, while those were normal in patients with MCD which has a more benign course. The findings reported herein are similar to the previous reports (6, 27, 28). Circulating TNF α is produced predominantly by cells of the monocytes/macrophage lineage (29). However, there were no differences in TNF α production by PBMC between patients and controls in our study. The previous study showed an increase in TNF α production by PBMC in patients with MN (6). Also no or a modest increase in TNF α production by PBMC was reported in diabetic patients (30, 31). These differences may be explained by the different dose of concanavalin A or subjects. When MC were incubated with PBMC supernatants from patients in our study, the TNF α levels in PBMC supernatants correlated with the production of MCP-1 or fibronectin by MC. Our observation suggests that TNF α production by PBMC in individual patients may affect the pathophysiology of their MC.

Our experiments demonstrated that PBMC from diabetic patients with or without nephropathy produced more MCP-1 when stimulated with concanavalin A. It is not obvious from this study whether the increased MCP-1 production by PBMC may facilitate the monocyte infiltration in the kidney, but it is reported that monocytes autonomously produce MCP-1 to regulate extravasation and activation of the same lineage (16). While the mechanisms involved in these results are unknown, monocyte chemotaxis and activation was shown to be induced by advanced glycosylation end-products which were increased in patients with DN (32, 33). On the other hand, our data showed that PBMC supernatants from patients with MCD or MN decreased MCP-1 production by MC. It is now unclear whether this suggests the presence of inhibitors such as soluble immune response suppressor in PBMC supernatants (3).

In these experiments, serum fibronectin levels were higher in patients with MN, not in those with MCD, while culture supernatants of PBMC from patients failed to increase the fibronectin production by MC. MN is characteristic of the glomerular basement membrane thickening and slow progression into renal failure. It is unclear whether this increase may reflect the ECM protein accumulation in the thickened glomerular capillary wall in MN or the progression of renal disease. On the other hand, our data showed no increase in serum fibronectin levels in patients with DN. It has been observed that plasma fibronectin levels were increased in diabetic patients with retinopathy or microalbuminuria

(24, 34), but a conflicting result was also reported (35). Therefore, it is suggested that urine fibronectin levels may be a useful indicator for DN (36).

In our results, effects of PBMC supernatants on the thymidine incorporation in MC were not different between patients and controls. The thymidine incorporation in MC was significantly decreased by sera from patients with DN and MCD. It tended to be decreased by sera from those with MN, but no significant difference was found. However, it cannot be clarified from this study whether it may be a common phenomenon by some serum factor(s) such as high concentrations of low density lipoprotein, TNF α or low concentrations of protein in nephrotic syndrome. It is known that the mesangial proliferation is not the characteristic feature in all of these diseases. Patients with nephrotic syndrome including MCD have high serum concentrations of low density lipoprotein which was observed to inhibit thymidine incorporation by MC (37). TNF α which has an antimitogenic activity in human MC (22) was increased in sera of patients with MN in this study. Our results in DN may be due to high glucose concentrations or the presence of nonenzymatic glycosylation endproduct in sera of the patients with DN, in line with the previous reports (31, 38, 39).

In summary, serum TNF α or fibronectin levels were increased in patients with MN that is known to progress to renal failure. Production of MCP-1 was increased by PBMC obtained from diabetic patients with or without nephropathy. Further studies may be helpful to clarify the meanings of these findings.

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