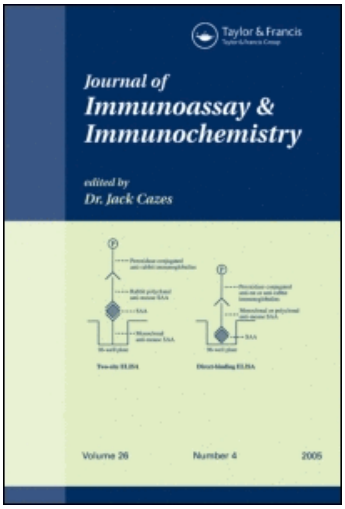

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MULTIPLEX BEAD ANALYSIS OF URINARY CYTOKINES OF TYPE 2 DIABETIC PATIENTS WITH NORMO- AND MICROALBUMINURIA

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□ *Renal damage of diabetic patients is linked to chronic low-grade inflammation. The aim of this study was to analyze urinary concentrations of 27 cytokines in type 2 diabetic patients with normo- and microalbuminuria by multiplex bead immunoassay. Urinary levels of IL-8, IP-10, MCP-1, G-CSF, EOTAXIN, RANTES, and TNF-α in microalbuminuric patients were significantly increased compared to those in normoalbuminuric patients ($p < 0.05$) or controls ($p < 0.05$). GM-CSF, MIP-1α, and MIP-1β levels were more elevated in microalbuminuric patients than in controls (both $p < 0.05$). IP-10 and MCP-1 levels were correlated with urinary albumin excretion rate ($r = 0.668$ and 0.544, both $p < 0.01$), and estimated glomerular filtration rate ($r = -0.454$ and -0.418, both $p < 0.05$). EOTAXIN, GM-CSF, IP-10, MCP-1, and RANTES levels were correlated with HbA1c ($r = 0.457$, 0.466, 0.678, 0.567, 0.542, respectively, $p \leq 0.01$). These results indicate that urinary cytokine determination might be useful for the early diagnosis and management of patients with diabetic nephropathy.*

Keywords chemokines, cytokines, diabetes, inflammation, microalbuminuria, normoalbuminuria

INTRODUCTION

Diabetic nephropathy (DN) is one of the most common microvascular complications of diabetes, and it is the major cause of end-stage renal disease worldwide.[1] The earliest clinical sign of DN is an elevated urinary albumin excretion, referred to as microalbuminuria. Persistent microalbuminuria in diabetic patients has been recognized not only as a

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predictor of progression of DN but also as a powerful risk factor for cardiovascular disease.[2,3]

There is increasing evidence that renal damage of diabetic patients is linked to chronic low-grade inflammation. Dalla Vestra et al. reported that type 2 diabetic patients with macroalbuminuria exhibited the highest levels of diverse, acute-phase markers of inflammation, including C-reactive protein, amyloid A, fibrinogen, and IL-6 in serum compared to those with normoalbuminuria and microalbuminuria.[4] Glomerular macrophage infiltration has been observed in kidney biopsy specimens from diabetic patients with glomerulosclerosis or in the streptozotocin-induced diabetic rat model.[5,6] Elevated pro-inflammatory cytokines, such as IL-1, IL-6, and IL-18, which are released by the infiltrating macrophages, and monocyte chemoattractant protein (MCP)-1, a chemokine that is known to affect the accumulation and function of macrophages, in serum or urine were correlated with the severity of DN, as demonstrated by increased urinary albumin excretion.[7–9]

Multiplex bead immunoassay can detect simultaneously a number of cytokines in a single sample. In addition, the high sensitivity of this technique permits its utilization in biological fluids, such as urine, serum, or saliva, to detect low abundant cytokines therein. In the present study, we used multiplex bead immunoassay to detect 27 cytokines in urine obtained from type 2 diabetic patients with normo- and microalbuminuria. Our data could result in improved understanding of the mechanisms of renal damage in diabetic patients and may help in the diagnostics for early stages of DN.

EXPERIMENTAL

Subjects

All diabetic patients were from the Department of Endocrinology, First Hospital Affiliated, Medical School of Xi'an Jiaotong University, Shaanxi, China. Diabetes mellitus was diagnosed according to the 1999 World Health Organization criteria. Subjects with a current acute illness (including infectious diseases), diabetic acute complications, or blood pressure (BP) exceeding 160/100 mmHg were excluded from the study.

The study was carried out in 31 patients with type 2 diabetes subdivided into two groups according to the presence of persistent microalbuminuria: 16 patients with microalbuminuria (Group 2) and 15 patients with normoalbuminuria (Group 3). Persistent microalbuminuria was defined as a urinary albumin excretion rate (UAER) between 30–300 mg/day in least two of three 24-h overnight urine collections within one month of the

primary visit. Moreover, 16 sex- and age-matched nondiabetic volunteers were studied as a control group (Group 1).

Before enrolment in the study, all subjects signed an informed consent after explanation of the nature and possible consequences of the study. All experimental protocols were reviewed and approved by the hospital Human Ethics Review Committee and complied with the Declaration of Helsinki for Experimentation on Humans (1975 and revised in 1983).

Laboratory Procedures

For measurement of clinical parameters, venous blood samples were collected after an overnight fast. Fasting plasma glucose (FPG) was determined by the glucose oxidase method. Glycated hemoglobin (HbA1c) levels were determined by high-performance liquid chromatography (Bio-Rad D-10, Hercules, CA, USA) and albuminuria by radioimmunoassay method. Serum creatinine (SCr) was measured using an autoanalyzer (Hitachi 7170 biochemic analyzer, Japan). All the methods are standard procedures used in pathology laboratories. Estimated glomerular filtration rate (eGFR) was calculated using the modification of diet in renal disease (MDRD) formula: eGFR (mL/min per $1.73\,m^2$) $= 186 \times SCr^{-1.154} \times (age\ [years])^{-0.203} \times (0.742\ if\ female)$.[10] Diabetic retinopathy was defined as nonproliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR) on the basis of fundus examination and fluorescein angiography. NPDR is characterized by microaneurysms, hemorrhages, exudates, macular ischemia, macular edema on the retina, and PDR, characterized by abnormal new vessel formation on the retina, vitreous hemorrhage, and traction retinal detachment.

The first voided morning urine samples were obtained and centrifuged at 5000 rpm for 10 min. Supernatant was stored at $-80°C$ until needed. Twenty seven cytokines [IL-1β, -2, -4, -5, -6, -7, -8, -9, -10, -12p70, -13, -15, -17, IL-1 receptor antagonist (IL-1RA), tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), granulocyte-monocyte colony stimulating factor (GM-CSF), granulocyte-colony stimulating factor (G-CSF), platelet-derived growth factor (PDGF)-BB, basic fibroblast growth factor (b-FGF), vascular endothelial growth factor (VEGF), MCP-1, macrophage inflammatory protein (MIP) -1α, -1β, EOTAXIN, interferon-induced protein (IP) -10, and regulated upon activation, normal T-cell expressed and presumably secreted (RANTES)] in all urine samples were detected in duplicate using multiplex bead analysis (Bio-Plex Human Cytokine 27-plex panel, Bio-Rad, Hercules, CA). Intra-assay and interassay coefficient variations were below 15% and 20%, respectively. Standard curves were generated by using the reference cytokine sample supplied in the kit, and were used to calculate the cytokine concentrations in urine samples. Urinary

levels of cytokines were related to the concomitant urinary creatinine concentration content, which was measured using a commercial kit from R&D System (Minneapolis, MN, USA), in order to compensate for variations in urinary concentration, and were expressed as pg/mg Cr. The concentrations of cytokines that were below the limit of detection of the assay were given zero value of concentration.

Statistical Analysis

Data were analyzed using the statistical package for social sciences (SPSS; SPSS Inc., Chicago, IL, USA). Data are presented as mean ± standard error of the mean (SEM) for variables normally distributed or medians (25th percentile, 75th percentile) for variables not normally distributed. Normally distributed values were analyzed by independent samples Student's *t*-test or analysis of variance (ANOVA) followed by Bonferroni multiple comparison test. For non-normally distributed differences between group pairs were estimated by the Nemenyi test after the nonparametric Krusaki–Wallis test had revealed significant differences among the groups. Correlations among the variables were performed by Spearman correlation. The multivariate general linear model was performed with albuminuric stages (normoalbuminuria and microalbuminuria) as a fixed factor and HbA1c as a covariate to exclude the influence of HbA1c. A p value of less than 0.05 was considered statistically significant.

RESULTS

Clinical Characteristics of Subjects

Clinical characteristics of the three groups are summarized in Table 1. No significant difference was demonstrated for sex, age, body mass index (BMI), or systolic or diastolic BP levels among the three groups. The microalbuminuric group had a longer diabetic history than the normoalbuminuric group ($p = 0.038$). As expected, UAER levels were higher in microalbuminuric patients than those in normoalbuminuric patients ($p < 0.001$) or controls ($p < 0.001$), and FPG levels were significantly raised in normo- and microalbuminuric patients compared with controls ($p = 0.012$ and $p = 0.041$, respectively). Mean HbA1c concentrations were elevated in the microalbuminuric group compared with the normoalbuminuric group ($p = 0.033$). No significant differences were found for SCr levels between normo- and microalbuminuric groups, while eGFR levels were significantly decreased in the microalbuminuric group compared with the normoalbuminuric group [$(80.4 \pm 4.3)\,\mathrm{mL/min^{-1}}\ 1.73\,\mathrm{m^2}$ vs.

TABLE 1 Clinical Characteristics of the Study Subjects

Parameters	Group 1	Group 2	Group 3
Age (years)	56.1 ± 2.6	61.1 ± 2.7	59.9 ± 3.0
Sex (M/F)	8/8	8/8	8/7
Diabetic duration (years)	–	13.1 ± 1.8†	7.8 ± 1.6
BMI (kg/m^2)	22.9 ± 0.7	23.2 ± 1.1	22.5 ± 0.8
FPG (mmol/l)	5.4 ± 0.2	8.5 ± 0.8*	8.0 ± 0.3*
SBP (mmHg)	120.6 ± 2.1	128.9 ± 2.4	123.5 ± 3.2
DBP (mmHg)	74.7 ± 1.9	76.7 ± 1.3	78.6 ± 2.1
HbA1c (%)	–	9.8 ± 0.7†	8.0 ± 0.5
UAER (mg/d)	9.3 ± 1.2	78.9 ± 9.4**‡	10.8 ± 1.7
SCr (μmol/L)	–	79.6 ± 5.1	71.5 ± 4.0
eGFR (mL/min^{-1} 1.73 m^2)	–	80.4 ± 4.3†	96.9 ± 6.0
Diabetic retinopathy (NDR/NPDR/PDR)	–	2/12/2	9/6/0

Data were expressed as mean ± SEM. Group 1, non-diabetic controls; Group 2, type 2 diabetic patients with microalbuminuria; Group 3, type 2 diabetic patients with normoalbuminuria. BMI, body mass index; FPG, fasting plasma glucose; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, glycated haemoglobin; UAER, urinary albumin excretion rate; SCr, serum creatinine; eGFR, estimated glomerular filtration rate. Diabetic retinopathy is subdivided into no retinopathy (NDR)/ nonproliferative diabetic retinopathy (NPDR)/proliferative diabetic retinopathy (PDR). The number of subjects in each stage is listed in this table.

$^{*}p < 0.05$, $^{**}p < 0.001$ vs. Group 1; $^{\dagger}p < 0.05$, $^{\ddagger}p < 0.001$ vs. Group 3.

(96.9 ± 6.0) mL/min^{-1} 1.73 m^2, $p = 0.034$)]. Diabetic retinopathy was observed significantly more often in diabetic patients with microalbuminuria than patients with normoalbuminuria.

Urinary Cytokine Levels Measured by Multiplex-27 Bead Immunoassay

To confirm the ability of multiplex bead immunoassay to measure urinary cytokines, spike-recovery experiments were conducted. A known quantity of each analyte cytokine was added to 120 μL of a urine sample, and this spiked sample was used to determine the percentage recovery of each cytokine. The percentage recovery was excellent (90–136%) for IL-1RA, EOTAXIN, GM-CSF, G-CSF, IL-8, IL-13, IL-12p70, IL-15, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α, and VEGF, and it was above 70% for the other cytokines except for IL-9 (29%).

Based on the detection limits of each cytokine, cytokines IL-2, -4, -5, -6, -7, -9, -10, -12p70, -13, -15, -17, b-FGF, and IFN-γ levels were below the detectable limit or detected infrequently using this method in any subjects, including diabetic patients. IL-1RA, EOTAXIN, GM-CSF, IP-10, MCP-1, MIP-1α, MIP-1β, RANTES, TNF-α, and VEGF levels were detectable in almost all urine samples from diabetic patients and control subjects. PDGF-BB was detectable in 75% of diabetic patients and controls.

TABLE 2 Urinary Cytokine Levels (pg/mg Cr) Measured by Multiplex-27 Bead Immunoassay

Cytokines	Group 1	Group 2	Group 3
IL-1RA	117.7 (21.4, 787.7)	650.5 (185.2, 6772.6)	119.2 (5.3, 1538.4)
IL-8	UD	36.0 (0, 270.0)**‡	UD
EOTAXIN	6.3 (0.9, 34.2)	56.7 (2.7, 166.9)**‡	14.4 (1.2, 41.6)
G-CSF	UD	7.1 (0, 20.6)**‡	UD
GM-CSF	35.6 (2.3, 94.3)	144.1 (5.5, 342.1)*	55.2 (1.9, 98.0)
IP-10	22.7 (5.5, 76.0)	417.1 (124.1, 708.0)**‡	30.9 (10.9, 135.4)
MCP-1	31.6 (8.0, 87.0)	730.5 (28.5, 1882.9)**‡	48.0 (5.3, 159.2)
MIP-1α	3.9 (1.9, 11.4)	46.3 (14.7, 83.6)*	8.4 (2.8, 13.1)
MIP-1β	10.7 (2.0, 27.4)	33.3 (9.1, 119.9)*	22.0 (3.2, 46.8)
PDGF-BB	11.7 (0, 37.5)	46.9 (0, 175.2)	11.7 (0, 29.2)
RANTES	4.0 (0.2, 10.5)	25.8 (2.7, 57.5)*†	6.0 (0.4, 9.3)
TNF-a	13.5 (0.6, 39.6)	54.7 (1.7, 213.0)*†	11.8 (0.4, 46.0)
VEGF	82.9 (0.3, 310.7)	410.0 (0.7, 802.9)	41.5 (0.2, 205.7)

Data were expressed as medians (25th percentile, 75th percentile). UD: undetectable. Cytokines IL-1β, -2, -4, -5, -6, -7, -9, -10, -12p70, -13, -15, -17, b-FGF, and IFN-γ levels were not detectable or detected infrequently and are not shown in this table.

$^{*}p<0.05$, $^{**}p<0.01$, vs. Group 1; $^{\dagger}p<0.05$,$^{\ddagger}p<0.01$ vs. Group 3.

Urinary levels of detectable cytokines are summarized in Table 2. IL-8 and G-CSF were detectable in 70% and 56% of urine samples, respectively, from microalbuminuric patients but not in those from controls or normoalbuminuric patients (Table 2). For nondiabetic control subjects, the most predominant urinary cytokines (median >10 pg/mg Cr), were IL-1RA (117.7 pg/mg Cr), followed by VEGF (82.9 pg/mg Cr), GM-CSF (35.6 pg/mg Cr), MCP-1 (31.6 pg/mg Cr), IP-10 (22.7 pg/mg Cr), TNF-α (13.5 pg/mg Cr), and MIP-1β (10.7 pg/mg Cr).

No significant differences were found in concentrations of urinary cytokines between normoalbuminuric patients and controls. However, urinary levels of IL-8, IP-10, MCP-1, G-CSF, EOTAXIN, RANTES, and TNF-α in diabetic patients with microalbuminuria were significantly increased compared to patients with normoalbuminuria ($p<0.01$ for IL-8, IP-10, MCP-1, EOTAXIN; $p<0.05$ for G-CSF, RANTES, and TNF-α) or controls ($p<0.01$ for IL-8, IP-10, MCP-1; $p<0.05$ for EOTAXIN, G-CSF, RANTES, and TNF-α). GM-CSF, MIP-1α, and MIP-1β levels were elevated in microalbuminuric patients compared to controls ($p=0.012$, 0.035, 0.043, respectively), but not significantly ($p>0.05$) compared to normoalbuminuric patients.

To exclude the influence of HbA1c, all cytokines were compared between patients with normo- and microalbuminuria using the multivariate general linear model. Albuminuric stages (normoalbuminuria and microalbuminuria) were included as a fixed factor and HbA1c was a covariate. The results showed that the differences for IL-8, MCP-1, IP-10, EOTAXIN,

RANTES, and TNF-α levels between patients with normo- and microalbuminuria were still significant ($p < 0.01$ for IL-8 and IP-10; $p < 0.05$ for MCP-1, EOTAXIN, RANTES, and TNF-α) after adjusting for the effect of HbA1c.

Relationships Between Urinary Cytokine Levels and Clinical Parameters

Spearman correlation analyses were performed between urinary cytokines (IL-1RA, IP-10, MCP-1, MIP-1α, MIP-1β, EOTAXIN, GM-CSF, RANTES, TNF-α, and VEGF) and clinical data. The results demonstrated that urinary levels of IP-10 and MCP-1 were significantly correlated with UAER ($r = 0.668$, $P < 0.001$ and $r = 0.544$, $p = 0.002$), and eGFR ($r = -0.454$, $p = 0.01$ and $r = -0.418$, $p = 0.019$) in diabetic patients. EOTAXIN, GM-CSF, IP-10, MCP-1, and RANTES levels were positively correlated with HbA1c ($r = 0.457$, $p = 0.010$; $r = 0.466$, $p = 0.008$; $r = 0.678$, $p < 0.001$; $r = 0.567$, $p = 0.001$; $r = 0.542$, $p = 0.002$, respectively) in diabetic patients. No association was observed between urinary cytokine levels and age, BMI, BP, SCr, or duration of diabetes (time since initial diagnosis).

DISCUSSION

The present study demonstrated that IL-1RA, EOTAXIN, GM-CSF, IP-10, MCP-1, MIP-1β, RANTES, TNF-α, and VEGF were the predominant cytokines in human urine, and urinary levels of many cytokines were undetectable by multiplex bead immunoassay in control subjects. Cytokines are low-molecular-weight, soluble proteins that are reabsorbed generally in renal tubules, which could result in lower detectability of the cytokines in our study.

We observed that urinary levels of IL-8, IP-10, MCP-1, MIP-1α, MIP-1β, G-CSF, GM-CSF, EOTAXIN, RANTES, and TNF-α were increased in type 2 patients with microalbuminuria compared to those with normoalbuminuria or control subjects. All these factors possess pro-inflammatory activities. Previous studies have shown that urinary MCP-1 or IL-8 levels were elevated in type 2 diabetic patients with nephropathy as detected by ELISA method.[11,12] Urinary MCP-1 and IP-10 levels were found to be elevated in type 1 diabetic patients with microalbuminuria and are associated with their progressive decline in renal function.[13] Elevated levels of these factors in urine of diabetic patients with microalbuminuria support the hypothesis that inflammation plays a significant role in early renal injury of diabetic patients.

For DN patients, previous studies have shown that the elevated cytokine levels in urine do not correlate with those in serum,[14–18] which suggests that these cytokines can be produced in the kidneys. It is known that diverse intrinsic renal cells, such as endothelial, mesangial, glomerular, and tubular epithelial cells, are able to synthesize and release cytokines.[19–21] Moreover, in vitro studies have demonstrated increased expression of TNF-α or MCP-1 mRNA in glomeruli of diabetic rats.[22,23] Elevated cytokine production in the kidney and increased permeability of the glomerular filtration barrier to proteins may contribute to elevated urinary cytokine levels of microalbuminuric patients in this study.

Poor glycemic control is a predictor of the development of nephropathy in diabetic patients.[24] High glucose and advanced glycation end-products can promote the production of cytokines in human or mouse mesangial cells through a pathway involving activation of protein kinase C, increases in levels of oxidative stress, and the activation/nuclear translocation of the transcription factor nuclear factor-κB.[25,26] In the present study, EOTAXIN, GM-CSF, IP-10, MCP-1, and RANTES levels were positively correlated with HbA1c in all diabetic patients. These results suggest that prolonged hyperglycemia, leading to increased HbA1c, might induce these pro-inflammatory cytokines. Intensive therapy to achieve near-normal blood glucose levels might moderate the development or progression of renal inflammatory damage. For diabetic patients with normoalbuminuria, although hyperglycemia may stimulate production of cytokines in the kidney, normal kidney function might be able to prevent their glomerular filtration ($eGFR = 96.9 \pm 6.0\,mL/min^{-1}\ 1.73\,m^2$) or/and guarantee their reabsorption in tubuli, so in the present study, cytokine levels were not significantly elevated in the normoalbuminuric group compared with those in the control group.

UAER and eGFR, two important indicators of the degree of renal injury in diabetic patients, reflect the lesion of glomerular basement membrane and alteration of glomerular filtration rate, respectively. In the present study, MCP-1 and IP-10 were positively correlated with UAER and negatively correlated with eGFR. Plasma levels of MCP-1 and IP-10 have been reported to correlate with the severity of DN.[27] Detecting urinary levels of these cytokines can be an indicator of the progression of diabetic renal injury. An increase in urinary protein excretion can aggravate renal tubulointerstitial lesions and up-regulate expression of cytokines in tubule cells, consequently accelerating the progression of DN.[28] Another possibility is that IP-10 and MCP-1 can directly affect the barrier function of the glomerular capillary.

Finally, two study limitations are acknowledged. We did not evaluate the time-dependent changes of urinary cytokine levels during a follow-up period. Cytokine levels may be modified by certain medications, such as renin

angiotensin (RAS) blockers, which are reported to inhibit the production of cytokines and provide renal protection for diabetic patients.[29] Furthermore, the number of investigated patients was relatively small. Therefore, additional study by long-term observation with more patients is required to elucidate more precisely whether the change of urinary cytokine levels might be induced by the RAS blockers.

CONCLUSIONS

In summary, using multiplex bead immunoassay, we observed elevated urinary levels of cytokines IL-8, IP-10, MCP-1, MIP-1α, MIP-1β, EOTAXIN, TNF-α, RANTES, G-CSF, and GM-CSF in diabetic patients with microalbuminuria. Many cytokine levels were related with the state of glycemic control. MCP-1 and IP-10 levels were correlated with the progression of albuminuria, which might be useful for early diagnosis of diabetic patients with nephropathy, especially when monitoring development and evolution of their disease. These elevated cytokines may be novel potential therapeutic targets, and inhibition of their production might be an efficacious treatment for renal damage secondary to diabetes mellitus in the future.

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