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Serum IL-9, IL-17, and TGF- β levels in subjects with diabetic kidney disease (CURES-134)

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Abstract

The role of inflammation in both diabetes and diabetic kidney disease (DKD) is becoming more widely accepted. However, the role of recently characterized T cell cytokines interleukin (IL)-9 and IL-17 in diabetes and especially DKD is less well studied. Transforming growth factor beta (TGF- β) controls the secretion of both of these cytokines. In this study, we estimated the levels of IL-9, IL-17, and TGF- β in the serum of subjects with normal glucose tolerance (NGT = 88) and subjects with type 2 diabetes without (diabetes mellitus (DM) = 65) and with DKD (DKD = 97) using enzyme-linked immunosorbent assay (ELISA), and we correlated these levels with the clinical risk factors of diabetes and DKD. IL-17 levels showed a serial decline and TGF- β levels showed a serial increase from NGT to DM to DKD ($p < 0.001$). However, the IL-9 levels were significantly reduced in the DM group compared to the NGT and DKD group ($p < 0.001$). While TGF- β and IL-17 showed a positive and negative correlation, respectively, with fasting and postprandial glucose levels and glycated hemoglobin (HbA1c), IL-9 showed positive correlation with urea and microalbuminuria. Apart from pro-inflammatory cytokines, T helper (Th) cytokines might play an important role in insulin resistance and DKD.

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Authors' contribution

V.A., V.M., and S.B. conceived and designed the experiments. R.V. and G.A. performed the experiments. R.V., M.D., V.A., G.A., and V.S. analyzed the data. R.V. and V.A. wrote the paper.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.cyto.2014.10.009>.

Keywords

Diabetic kidney disease; Nephropathy; IL-9; IL-17; TGF- β and insulin resistance

1. Introduction

Diabetic kidney disease (DKD) occurs in about 35–40% of patients with both type 1 and type 2 diabetes [1]. The classic view of metabolic and hemodynamic alterations as the main causes of renal injury in DKD has been transformed significantly [2]. One of the most important recent advances in our understanding of DKD is the participation of inflammatory processes in the pathogenesis of the disease [2]. Increased infiltration of activated T cells and aberrant expression of T cell cytokines have been reported in DKD [3]. However, no data are currently available on the recently characterized T cell-derived cytokine interleukin (IL)-9 in DKD. IL-9, initially recognized as a type 2 T helper (Th2) cytokine, was recently attributed to a novel CD4 T cell subset termed Th9 [4]. However, IL-9 can also be secreted by Th17 cells, and it may mediate aspects of the pro-inflammatory activities of Th17 cells [4]. The production of both IL-9 and IL-17 by T cells is under the control of TGF- β [4]. A resurgence of interest in IL-9 and IL-17 has been spurred by recent work demonstrating a role for IL-9 in obesity [5,6], insulin resistance [6], and kidney disease in general [7,8]. In the present study, our objective was to estimate the levels of IL-9 along with IL-17 and transforming growth factor beta (TGF- β) in subjects with type 2 diabetes with DKD and to correlate these levels with the clinical risk factors associated with diabetes mellitus (DM) (IR and glycated hemoglobin (HbA1c)) and with DKD (blood urea, serum creatinine, estimated glomerular filtration rate (eGFR), and microalbuminuria).

2. Methods

2.1. Study population

Study participants were recruited from the Chennai Urban Rural Epidemiology Study (CURES), a large epidemiological study conducted on a representative population of Chennai (formerly Madras City in southern India). The exclusion criteria were patients with type 1 diabetes and those previously diagnosed with urolithiasis, recent or current viral hepatitis or cirrhosis of liver, congestive heart failure, chronic lung disease, or acute or chronic infections. Institutional ethical committee approval was obtained from the Madras Diabetes Research Foundation Ethics Committee (Ref No-MDRF-EC/SOC/2009//05), and written informed consent was obtained from all the study participants. The study was conducted as per the Declaration of Helsinki.

2.2. Anthropometric and biochemical parameters

Anthropometric measurements including height, weight, and waist circumference were obtained using standardized techniques. The body mass index (BMI) was calculated as the weight in kilograms divided by the square of height in meters. Fasting plasma glucose (FPG) (the glucose oxidase–peroxidase method), serum cholesterol (the cholesterol oxidase–peroxidase–amidopyrine method), serum triglycerides (the glycerol phosphate oxidase–peroxidase–amidopyrine method), high-density lipoprotein cholesterol (HDL-C) (a direct

method using polyethylene glycol-pretreated enzymes), urea, and creatinine (Jaffe's method) were measured using a Hitachi-912 AutoAnalyzer (Hitachi, Mannheim, Germany). Glycated hemoglobin (HbA1c) was estimated by high-pressure liquid chromatography using a variant machine (Bio-Rad, Hercules, CA, USA). The intra- and inter-assay coefficient of variation for the biochemical assays ranged between 3.1% and 5.6%.

2.3. Diagnosis of DKD

Urinary albumin concentration was measured in a fasting sample using an immunoturbidimetric assay (Hitachi 902 AutoAnalyzer; Roche Diagnostics). Subjects were classified based on albumin excretion per milligram of creatinine as normoalbuminuric ($< 29 \mu\text{g}$), microalbuminuric ($30\text{--}229 \mu\text{g}$), or albuminuric/overt nephropathy ($\geq 300 \mu\text{g}$). Data on serum creatinine, age, and sex were used to calculate the eGFR using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [9].

2.4. Estimation of serum cytokine levels

The levels of cytokines IL-9 (ebiosciences, USA), IL-17 (R&D System, USA), and TGF- β (R&D System, USA) were estimated by enzyme-linked immunosorbent assay (ELISA) as per the instructions of the kit. In brief, cytokine-specific monoclonal antibody was coated onto microplates followed by the addition of standards and samples. After washing and blocking, enzyme-linked monoclonal antibodies were added. After washing, the substrate solution was added and the absorbance was read at 450 nm. The intra- and inter-assay coefficients of variation for multiplex assay were $<5\%$ as determined in our laboratory. The lowest detection limit for IL-9, IL-17, and TGF- β were 1, 2, and 4 pg/ml.

2.5. Sample size calculation

Initially, 20 NGT, 20 DM, and 20 diabetic nephropathy (DN) subjects were screened for the serum cytokines studied. On the basis of the preliminary results, with a confidence interval of 95%, an estimated p -value <0.05 , and a power of 80%, a sample size of 60 per group was calculated. However, we increased the numbers in each group to account for the wide variation generally seen among serum biomarkers.

2.6. Statistical analyses

Student's t -test was used to compare groups for continuous variables that followed normal distribution, whereas the χ^2 test or Fisher's exact test (as appropriate) was used to compare proportions. The Kruskal–Wallis test was used for multiple parameters that did not show normal distribution. Spearman's correlation analysis and multivariate logistic regression analysis were used to determine the association of cytokines with clinical parameters and study groups, respectively. Multiple comparisons were corrected using Holm's correction for each set of analysis. All the analyses were conducted using SPSS statistical package (Version 20.0; SPSS, Chicago, IL, USA) and a p -value <0.05 was considered significant.

3. Results and discussion

Table 1 presents the clinical and biochemical characteristics of the study subjects. The age was significantly higher in the DKD group compared to the NGT and DM groups ($p <$

0.0001). BMI showed a linear increase from NGT to DM to DKD ($p = 0.003$). Subjects with DKD had higher blood pressure (BP) (both systolic and diastolic) compared to those of the NGT and DM groups ($p < 0.001$). Subjects with DKD had significantly lower levels of low-density lipoprotein (LDL) cholesterol ($p < 0.0001$) and eGFR ($p = 0.003$) and higher levels of triglycerides ($p < 0.0001$), while the HDL-C levels were not significantly different between the groups. Subjects with DKD also had significantly elevated levels of postprandial plasma glucose (PPPG) ($p < 0.0001$), glycated hemoglobin ($p < 0.001$), blood urea ($p < 0.0001$), serum creatinine ($p = 0.003$), and microalbuminuria ($p < 0.001$). Based on eGFR values and as per CKD-EPI criteria, 45 (47%) of the subjects with DN were in stage 1 (eGFR ≥ 90), 31 (32%) in stage 2 (eGFR = 60–89), 17 (18%) in stage 3 (eGFR = 30–59), and three (3%) in stage 4 (eGFR = 15–29) of CKD, and none were in stage 5 (eGFR ≤ 15).

Fig. 1 shows the levels of serum cytokines IL-17, TGF- β , and IL-9 in the study groups. Fig. 1a shows that the IL-17 levels were significantly lower in the DM and DKD groups compared to the NGT group (GM (range): NGT 94.7 (69.2–120.2) pg/ml vs. DM 59.8 (54.2–65.4) pg/ml vs. DKD 67.4 (62.9–72.0) pg/ml; $p < 0.001$). Fig. 1b shows that TGF- β levels were progressively higher from the NGT to DM to DKD groups (GM (range): NGT 264.6 (156.1–373.0) pg/ml vs. DM 1334 (1079–1588) pg/ml vs. DKD 2410 (2088–2733) pg/ml; $p < 0.001$). Fig. 1c shows that IL-9 levels were significantly lower in DM compared to both the NGT and DKD groups (GM (range): NGT 3.53 (2.54–4.52) pg/ml vs. DM 1.43 (1.19–1.68) pg/ml vs. DKD 3.15 (2.46–3.84) pg/ml; $p < 0.001$). The serum levels reported in this study are in good agreement with two recent clinical studies wherein serum IL-9 levels in chronic lymphoblastic leukemia [10] and urticaria [11] were reported.

Spearman's correlation analysis was carried out between the mean cytokine levels and various biochemical parameters. As presented in Table S.1, age ($r = 0.49$; $p < 0.001$), diastolic BP ($r = 0.21$; $p = 0.002$), and serum triglycerides ($r = 0.22$; $p = 0.001$) showed a positive correlation with TGF- β , whereas serum cholesterol ($r = -0.15$; $p = 0.02$) and LDL cholesterol ($r = -0.26$; $p < 0.001$) showed a negative correlation with TGF- β . Blood urea (IL-9: $r = 0.19$, $p = 0.009$; TGF- β : $r = 0.32$, $p < 0.001$) and microalbuminuria (IL-9: $r = 0.189$; $p = 0.015$; TGF- β : $r = 0.36$, $p < 0.001$) showed a positive correlation with both TGF- β and IL-9. FPG (IL-17: $r = -0.21$, $p < 0.001$; TGF- β : $r = 0.59$; $p < 0.001$), PPBG (IL-17: $r = -0.18$, $p = 0.006$; TGF- β : $r = 0.55$, $p < 0.001$), HbA1c (IL-17: $r = 0.145$, $p = 0.022$; TGF- β : $r = 0.57$, $p < 0.001$), and systolic BP (IL-17: $r = -0.13$, $p = 0.046$; TGF- β : $r = 0.36$, $p < 0.001$) showed a positive correlation with both TGF- β and IL-17.

Multivariate logistic regression analysis was carried out using disease phenotype as the dependent variable and log-transformed cytokine levels as the independent variables. Table S.2 shows that TGF- β (OR = 1.003; 95% CI = 1.001–1.005; $p = 0.001$) alone showed a significant positive association with DM even after adjusting for age and gender, while IL-9 (OR = 0.48; 95% CI = 0.23–0.99; $p = 0.046$) showed a significant negative association with DM after adjusting for age and gender. Both IL-17 (OR = 1.03; 95% CI = 1.002–1.06; $p = 0.03$) and IL-9 (OR = 1.5; 95% CI = 1.05–2.14; $p = 0.03$) showed a significant association with DKD, after adjusting for age and gender.

To the best of our knowledge, this is the first report to document the levels of IL-9 in DKD in a clinical setting. DKD is characterized by chronic, low-grade, nonspecific inflammation. However, the contribution of T cell-derived cytokines towards DKD-specific inflammation is less well known. This study shows that T cell-derived cytokines, especially IL-9 and IL-17, may play a role in the disease process. IL-9 can function as both a positive and negative regulator of immune responses. In general, it seems that IL-9 has detrimental roles during allergy and autoimmunity [5]. However, during parasitic infections, IL-9 can help clear the pathogen, and during skin transplantation, IL-9 can promote the maintenance of a tolerant environment [5]. Recently, serum IL-9 levels were found to decrease in patients who underwent OK432-stimulated dendritic cell transfer into hepatocellular carcinoma during transarterial embolization [12]. The role played by IL-9 in metabolic diseases is largely unknown. Beriou et al. first reported an increased frequency of IL-9 + IL-17 + memory CD4 + T cells in subjects with type 1 diabetes mellitus (T1DM) [13]. In a subsequent study, gene expression analysis carried out on T1DM monozygotic quadruplets reconfirmed the involvement of IL-9 signaling in the pathogenesis of pancreatic β cell destruction [14]. However, whether the same holds good for type 2 diabetes mellitus (T2DM) is largely unknown. In a recent study, IL-9 levels were found to be undetectable in subjects with NGT, but these levels were significantly elevated in obese subjects with and without T2DM [5]. However, even among obese subjects, the levels became undetectable following gastric bypass surgery [5]. However, Chen et al. reported no difference in the serum concentration of IL-9 between subjects with NGT and T2DM (with DN) [15]. Thus, more clinical studies are needed to clarify the role of IL-9 in type 2 diabetes and its microvascular complications. With respect to decreased serum IL-17 levels, our results are in agreement with our previous report on metabolic syndrome (MS) [6] and with the report by Arababadi et al. [8].

4. Conclusion

The major limitation of our study is its cross-sectional nature, which suggests that no cause-and-effect relationship can be drawn. Further, as this is a serological study, characterizing Th9 and Th17 cells in the study subjects is beyond the scope of this paper. The strength of this study is that it is one of the first to report the association of these cytokines with type 2 diabetes and with DKD. Despite the study being cross-sectional in nature, it gains importance in the context of it being conducted in an ethnic population of Asian Indians who are at a high risk of diabetes and DKD where no report on this topic is currently available. In summary, this study suggests a linear negative association between IL-17 and DKD and a positive association between TGF- β and DKD. The association between IL-9 and DKD was rather complex as it was reduced in DM, but it was of normal levels in those with DKD. The reason for this is not clear. Future studies should attempt to elucidate the role of IL-9, IL-17, and TGF- β in DKD in general, and insulin resistance in particular.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations:

NGT	normal glucose tolerance
DM	diabetes mellitus (type 2)
IL-9	interleukin 9
IL-17	interleukin 17
TGF-β	transforming growth factor beta
HbA1c	glycated hemoglobin

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Fig. 1.

Interleukin (IL)-9, IL-17, and TGF- β serum cytokine levels in subjects with NGT, DM, and DKD: (a) IL-17, (b) TGF- β , and (c) IL-9. The geometric mean is represented by the horizontal bars. The p -values were calculated by Kruskal–Wallis one-way analysis of variance. A p -value <0.05 was considered significant.

Table 1

Clinical and biochemical characteristics of the study subjects.

Clinical parameters	NGT* (n = 88)	DM* (n = 65)	DKD* (n = 97)	p-Value
Age (years)	37.9 ± 12.4	51.3 ± 12.2	59.3 ± 12.1	<0.0001
Gender (M/F)	35/53	38/27	57/40	NS
BMI* (kg/m ²)	24.2 ± 4.9	26.3 ± 4.5	26.9 ± 5.5	0.0029
Systolic BP* (mm Hg)	116 ± 19	129 ± 17	138 ± 15	<0.0001
Diastolic BP* (mm Hg)	74 ± 12	77 ± 8	83 ± 9	<0.0001
FPG* (mg/dl)	82 ± 7	180 ± 35	166 ± 57	<0.0001
PPBS* (mg/dl)	102 ± 22	199 ± 76	243 ± 81	<0.0001
HbA1c levels* (%)	5.5 ± 0.5	7.6 ± 1.7	9.5 ± 7.0	<0.0001
Cholesterol (mg/dl)	175 ± 38	164 ± 41	158 ± 39	0.0114
Serum triglycerides (mg/dl)	114 ± 54	136 ± 55	160.7 ± 79	<0.0001
HDL* cholesterol (mg/dl)	42.6 ± 10.2	40.7 ± 8.6	41.2 ± 10.5	0.5297
LDL* cholesterol (mg/dl)	109.3 ± 33.4	90.0 ± 26.3	84.1 ± 31.1	<0.0001
Serum creatinine (mg/dl)	0.8 ± 0.1	0.8 ± 0.1	1.0 ± 0.4	0.0031
Microalbuminuria (mg/dl)	15.3 ± 36.8	6.8 ± 5.7	116.1 ± 81.4	<0.0001
eGFR*	96.0 ± 20.8	101.2 ± 20.4	86.9 ± 32.2	0.0031
Retinopathy	Nil	Nil	54.6%	NA

* NGT - normal glucose tolerance, DM - type 2 diabetes, DKD - diabetes kidney disease, BMI - body mass index, BP - blood pressure, FPG - fasting plasma glucose, HbA1c - glycated hemoglobin, HDL - high-density lipoprotein, LDL low-density lipoprotein, VLDL - very low-density lipoprotein, Malb - microalbuminuria, PPBS - postprandial blood sugar, GFR - glomerular filtration rate.