RELATIONSHIP OF PROINFLAMMATORY CYTOKINES WITH MICROALBUMINURIA IN PATIENTS OF TYPE 2 DIABETES MELLITUS- IMPLICATIONS IN THE PATHOGENESIS OF DIABETIC NEPHROPATHY

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ABSTRACT

BACKGROUND

Type 2 diabetes mellitus is frequently associated with an increased inflammatory state. However, the relationship between low grade inflammation and diabetic nephropathy still remains unclear with conflicting results. We therefore intended to analyse the inter-relationships between pro inflammatory cytokines and renal functions with respect to the glycemic status in type 2 diabetes mellitus patients.

MATERIALS AND METHODS

89 type 2 Diabetes mellitus patients and 26 age matched healthy controls were studied (29 normoalbuminuric, 33 microalbuminuric and 27 proteinuric) and their levels of proinflammatory cytokines (IL-6 and TNF-α) were measured and correlated with albuminuria and glycosylated haemoglobin.

RESULTS

Urinary albumin excretion showed positive correlation with the levels of Interleukin-6 (r=0.711 P < 0.001), as well as Tumour Necrosis Factor Alpha (r=0.591; P < 0.005) in the diabetics. The glycosylated haemoglobin had a positive correlation with IL-6 (r=0.792, P <0.001) and Urinary albumin excretion rate (r=0.685, P < 0.001) and mild positive correlation with TNF-α (r=0.589, P <0.005). There was no such correlation observed among control subjects.

CONCLUSION

The study shows that pro inflammatory cytokines levels are elevated in early diabetic nephropathy and are independently associated with urinary albumin excretion thus it can be hypothesized that their local release play a role in the renal damage in the development of renal damage.

KEYWORDS

Interleukin-6, TNF-α, microalbuminuria, diabetic nephropathy, proinflammatory cytokines.


BACKGROUND

The major cause of morbidity in a chronic Type 2 diabetes mellitus patient is diabetic nephropathy and its complications. Being one of the leading cause of chronic renal failure, diabetic nephropathy has been the area of interest of most clinicians and clinical researchers. Various metabolic and hemodynamic factors play a key role in the development of diabetic nephropathy. However, the exact mechanism regarding the initiation and development of the disease still remains a major grey area. Many studies have investigated the role of low grade inflammation in the disease process.¹⁻³ The pro-inflammatory cytokines that have been mostly associated with the generalized inflammatory status in diabetes mellitus is Interleukin-6 (IL-6) and Tumor Necrosis Factor Alpha (TNF-α). These pleiotropic cytokines have major impact on immune modulation of various cells and tissues of our body. Interleukin-6 has been shown to exert its effect on extracellular matrix dynamics at mesangial and podocyte levels, along with stimulation of mesangial cell proliferation, increased expression of fibronectin, and enhancement of endothelial permeability. Its effect is augmented by TNF-α.⁴ So there are indications that low grade inflammation plays a vital role in kidney injury in Type 2 DM. However, the results of similar such studies⁵⁻⁶ are conflicting and the question still remains unanswered regarding the proper role of these pro-inflammatory cytokines play in the pathogenesis of early stages of diabetic nephropathy. The aim of this study was to investigate the relationships between proinflammatory cytokines, glycemic indicators and renal functions in patients of type 2 diabetes mellitus.
MATERIALS AND METHODS

Study Design-
Data was collected over a period of six months from patients with type 2 Diabetes mellitus attending the endocrinology outpatient department of Institute of Post Graduate Medical Education and Research (IPGMER), Kolkata. Exclusion criteria for patients were acute illness in the recent past week, infectious diseases, any existing malignancy and active cardiovascular and immunological disorders. Patients having hypertension (BP more than 140/90 mm of Hg) or history of smoking were also excluded from the study. The exclusion criteria also included patients who had an existing renal insufficiency (Serum Creatinine more than 2.0 mg/dl).

A total of 115 patients were studied among which, the diabetics consisted of 3 age matched groups who were classified according to the degree of 24-hour urinary albumin excretion rate (UAE). The groups were normal albuminuria ((UAE consistently lower than 30 mg/day) n=29), microalbuminuria ((UAE between 30 mg/day and 300 mg/day) n=33) and macroalbuminuria ((UAE more than 300 mg/day) n=27). 26 healthy age matched individuals were included in the study as a control group. The controls were taken from relatives of patients attending the endocrinology, neurology and psychiatry OPD who did not have any diagnosed disease or symptoms. Written informed consent was obtained from all the participants involved in the study.

Blood Sample Collection
Venous blood samples were taken in sterile tubes from patients before breakfast in the morning (between 8 AM and 11 AM), after an 8- to 12-hour overnight fasting. Samples were centrifuged at 3000 rpm for 10 minutes at 4°C, and then stored at -20°C until the assay procedures. Fasting plasma glucose (FPG) level was measured by auto analyser (Randox Daytona) by glucose oxidase method using commercially available kit. Serum creatinine was estimated using autoanalyser (Randox Daytona) using commercial kit by Jaffe's method.

Estimation of Urinary Albumin Excretion
Urinary microalbumin was estimated with commercially available microalbumin kit for autoanalyser (Randox Daytona). Urinary albumin excretion (UAE) was estimated from a mean of two 24-hour sterile urine samples collected from subjects and expressed in mg/day.

Pro Inflammatory Cytokines
The serum levels of Interleukin 6 and TNF α were measured by ELISA. Samples were measured in triplicate using commercially available human Elisa kits (Abcam). The wells were washed using an automated washer (Fisher Scientific) and reading was taken in an automated Elisa reader (Tecan). The intra and inter-assay CVs are mentioned in Table 1.

Statistical Analysis
Data has been expressed as mean ± SD or as median and range as applicable. The data was checked for normal distribution using Kolgomorov-Smirnov test, taking P<0.0001 as significant. Students "t" test and Man-Whitney U was used to calculate differences in the parameters between the groups as applicable. The other continuous variables which have been classified according to the degree of albuminuria was compared using 1-way analysis of variance (ANOVA) with Tukey's post hoc test and Kruskal-Wallis' test with Dunn's nonparametric comparison between groups as applicable. Correlation between various parameters were done using Spearman's method with two tailed test for significance. The statistical significance was accepted taking P value<0.05. Statistical analyses of data were performed using the SPSS software version 19.

RESULTS

Comparison of Diabetics and Controls-
The clinical and demographic features of the diabetics were compared with those of the age matched healthy control subjects. Table 2 shows significant differences of means, in case of FPG, HbA1c, Urinary Albumin Excretion, IL6 and TNF α between the diabetics and the controls. However, there are no differences in Age, BMI and BP between the two groups.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Diabetic Patients</th>
<th>Age Matched controls</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>89</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>59 ± 9</td>
<td>57 ± 7</td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td>24 ± 6</td>
<td>22 ± 7</td>
<td></td>
</tr>
<tr>
<td>Diabetes duration (yrs.)</td>
<td>11.9 ± 3.1</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>SBP/DBP in mm of Hg</td>
<td>126 ± 8/82 ± 6</td>
<td>125 ± 5/ 76 ± 8</td>
<td></td>
</tr>
<tr>
<td>FPG (mg/dl)</td>
<td>148 ± 14</td>
<td>82 ± 12</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HbA1c (gm%)</td>
<td>8.4 ± 2.9</td>
<td>5.5 ± 0.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum Creatinine (mg/dl)</td>
<td>1.2 ± 0.2</td>
<td>0.9 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>Urinary AE(mg/day)</td>
<td>239 (6 to 1556)</td>
<td>5.4 (0 to 49)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>7.9 (4.8 to 11.1)</td>
<td>3.6 (2.3 to 6.2)</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>TNF α (pg/ml)</td>
<td>14.7 (8.5 to 19.6)</td>
<td>9.9 (8.4 to 10.6)</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Table 2. Comparison of Healthy Controls and Diabetic Patients Based on Demographic Features and Investigation Parameters
Data represented as Mean ± SD or as Median (Range). The P values have been shown for the parameters whose differences are statistically significant.

The comparative study of the diabetic group stratified according to UAE-
In Table 3, the parameters of the patients that have been stratified on the basis of urinary albumin excretion into 3 groups has been compared. The normoalbuminuric (n=29), microalbuminuric (n=33) and Proteinuric (n=27) group showed statistically significant differences of means in HbA1c, and the pro inflammatory cytokines (IL-6 and TNF-α), with the proteinuric group having the highest levels. The P values of inter-group comparisons have been shown in the table. However, the groups did not differ in Age, BP and Duration of diabetes.

<table>
<thead>
<tr>
<th>Patient Parameters</th>
<th>Normoalbuminuria (NA) ACR (&lt;30 mg /day) n=29</th>
<th>Microalbuminuria (MA) ACR (30-300 mg /day) n=33</th>
<th>Macroalbuminuria (M) ACR (&gt;300 mg /day) n=27</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs.)</td>
<td>58 ± 6</td>
<td>61 ± 5</td>
<td>55 ± 9</td>
<td></td>
</tr>
<tr>
<td>Diabetes duration (yrs.)</td>
<td>11.1 ± 4.2</td>
<td>9.9 ± 6.8</td>
<td>12.4 ± 4.1</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>24 ± 5</td>
<td>23 ± 4</td>
<td>25 ± 5</td>
<td></td>
</tr>
<tr>
<td>Mean BP (mm of Hg)</td>
<td>110 ± 8</td>
<td>108 ± 9</td>
<td>109 ± 10</td>
<td></td>
</tr>
<tr>
<td>FPG (mg/dl)</td>
<td>125.3 ± 42.3</td>
<td>156.5 ± 39.2</td>
<td>164.3 ± 45.8</td>
<td>&lt;0.05 M vs. NA</td>
</tr>
<tr>
<td>HbA1c (gm %)</td>
<td>7.1 ± 0.7</td>
<td>8.7 ± 0.6</td>
<td>9.6 ± 0.7</td>
<td>&lt;0.001 M vs. NA</td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>0.9 ± 0.1</td>
<td>1.0 ± 0.32</td>
<td>1.2 ± 0.2</td>
<td>&lt;0.005 M vs. MA</td>
</tr>
<tr>
<td>Urinary ACR (mg/day)</td>
<td>17 (6 to 28)</td>
<td>159 (49 to 286)</td>
<td>667 (412 to 1556)</td>
<td>&lt;0.003 M vs. NA</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>5.8 (5.1 to 7.7)</td>
<td>6.7 (4.8 to 8.1)</td>
<td>9.5 (8.4 to 11.1)</td>
<td>&lt;0.03 M vs. MA</td>
</tr>
<tr>
<td>TNF α (pg/ml)</td>
<td>12.8 (8.5 to 17.8)</td>
<td>13.8 (9.6 to 19.6)</td>
<td>14.1 (10.1 to 18.9)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Parameters of Diabetic Patients Classified on the Degree of Albuminuria

Relationship between Proteinuria, Glycemic Status and Proinflammatory Cytokines.
Spearman’s method was used to calculate the correlation of various parameters among the diabetic and control group.

The analysis in the diabetic group showed a positive coefficient between Albuminuria and IL-6 (r=0.711 P <0.001) A mild positive correlation coefficient was also found between UAE and TNF α (r=0.591; P <0.005). The Hba1C did show a strong positive correlation with IL-6 (r=0.792, P <0.001) and UAE (r=0.685, P <0.001) and mild positive correlation with TNF α (r= 0.589, P <0.005).

The control group did not show any correlation between Albuminuria, Hba1C and the Pro Inflammatory Cytokines.

DISCUSSION
This study demonstrated a significant association between the pro inflammatory cytokines and albuminuria in type 2 diabetes patients. Till now inflammation and diabetes have been proved to be causally associated with diabetic retinopathy, however the study of such similar causal association in case of diabetic nephropathy has led to conflicting results.6,6 In our study the strong association of IL-6 and TNF α with the glycemic status as wells UAE in early stages of diabetic nephropathy is of much importance as we ruled out confounding factors that might interfere with such association in our exclusion criteria of patients viz. Acute illness in the recent past week, infectious diseases, any existing malignancy and active cardiovascular and immunological disorders. Hypertension, history of smoking and an existing renal insufficiency. The pro inflammatory cytokines levels are higher in diabetic group of patients compared to the control group and among the diabetic group they are raised in group of patients having poor glycemic control as evident by its positive correlation with Hba1C.

We found that the concentrations of the pro inflammatory cytokine IL-6 and TNF α were significantly higher in the group of patients with frank proteinuria compared to n those with normoalbuminuria or microalbuminuria. Studies have demonstrated glomerular production of IL-6 and TNF α. The glomerular origin of IL6 has been demonstrated by in situ hybridization by Sukuzi et al.7 The glomerular production of TNF α has been shown by several studies.8-11 Therefore in the setting of diabetes, the possibility of a glomerular origin of these cytokines is hard to be overlooked. The phenomenon of glomerular as well renal interstitial infiltration by macrophages in patients of diabetic nephropathy is well known.12 Therefore those glomeruli may be responsible for the increased levels of cytokine production where there has been cessation of macrophage infiltration. The evidence of chronic inflammation in patients with kidney failure or uraemia has been indicated by several studies.13,14 Adipose tissue synthesize considerable amount of IL6 and TNF α which
should be noted in attributing the differences in these pro inflammatory cytokines due to increasing severity of diabetic nephropathy in the present study. However, in our current patient subgroups there was no difference in BMI hence our study supported a relationship between the low-grade inflammation and diabetic nephropathy in Type 2 diabetics. There may be several possible mechanisms of the finding of an association between the pro inflammatory cytokines and albuminuria in the setting of type 2 diabetes. Pre-existing atherosclerosis may result in an inflammatory state and trigger the rise of inflammatory markers. The fact that microalbuminuria in both type 2 diabetes as well as in non-diabetics is associated with increased cardiovascular morbidity and mortality is clearly suggestive that atherosclerosis prevails in individuals with albuminuria. Elevated pro inflammatory cytokines can directly cause an alteration in glomerular function and thus can be causally involved in the development of albuminuria. There are various studies which has shown an elevated UAE in inflammatory diseases as well as in conditions such as AMI, surgery, brain injury, trauma and similar acute syndromes. The link between glomerular function and inflammatory cytokines is evident by experimental data which suggests that IL6 and TNFA induce glomerular infiltration by leukocytes. They exert a strong influence on glycosaminoglycans which are a vital component of renal vascular endothelium and the glomerular basement membrane. Thus, their involvement in the aetiology of microalbuminuria and other macrovascular diseases in type 2 diabetes patients is highly possible.

CONCLUSION
In addition to traditional and metabolic factors, inflammation may be an additional pathogenic mechanism in diabetic nephropathy as indicated by the association between inflammatory cytokines and UAE. The release of locally released cytokines such as of IL-6 and TNF-α may trigger the development of low grade inflammation that leads to the initiation of kidney damage in type 2 diabetics. Control and prevention of obesity and hyperglycaemia, antioxidants therapy and treatment with other anti-inflammatory reagents may prove beneficial in controlling the early progressive inflammatory response associated with microangiopathic renal lesions in diabetes and leaves a scope for further studies in this regard.

Limitations of the Study
This study does not directly prove the renal production IL-6 and TNF-α therefore further studies involving the expression of mRNA of these cytokines by real time PCR in type 2 diabetic patients is needed to confirm the intra renal production and their implication of the inflammatory pathogenesis of diabetic nephropathy.

REFERENCES