

Acute-Phase Markers of Inflammation and Glomerular Structure in Patients with Type 2 Diabetes

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Type 2 diabetes is frequently associated with an inflammatory status; the relationships between low-grade inflammation and diabetic nephropathy are still unclear. The aim of this study was to evaluate the relationships between acute-phase markers of inflammation, glomerular structure, and albumin excretion rate (AER) in type 2 diabetes. In 74 patients with type 2 diabetes (23 normoalbuminuric, 30 microalbuminuric, and 21 proteinuric) fibrinogen, serum amyloid A protein (SAA), C-reactive protein (CRP), and IL-6 were determined. AER was measured on three 24-h urine collections; GFR was measured by ⁵¹Cr EDTA plasma clearance. A kidney biopsy was performed, and mesangial fractional volume [Vv(mes/glom)] and glomerular basement membrane (GBM) width were estimated by electron microscopic morphometric analysis. CRP, fibrinogen, SAA, and IL-6 differed among groups, with proteinuric patients having the highest levels. SAA and fibrinogen correlated with AER ($P < 0.03$ and $P < 0.001$, respectively). GBM width and Vv(mes/glom) increased from normoalbuminuric to proteinuric patients [$P < 0.005$ normoalbuminuric and microalbuminuric versus proteinuric for GBM, $P < 0.01$ normoalbuminuric versus proteinuric for Vv(mes/glom)]. In patients with increased GBM width (>396 nm), CRP, SAA, and IL-6 were higher than in patients with normal GBM width ($P < 0.003$, $P < 0.004$, and $P < 0.0004$, respectively). GBM width was directly correlated with fibrinogen ($r = 0.33$, $P < 0.002$) and IL-6 ($r = 0.25$, $P < 0.05$). In conclusion, this study demonstrates that acute-phase markers of inflammation are associated with nephropathy status and GBM thickening, suggesting a role for inflammation in the pathogenesis of diabetic glomerulopathy.

J Am Soc Nephrol 16: S78–S82, 2005. doi: 10.1681/ASN.2004110961

Type 2 diabetes is frequently associated with an acute-phase reaction, suggestive of a low-grade inflammatory status (1,2). In fact, markers of acute-phase response, including serum amyloid A (SAA), C-reactive protein (CRP), and IL-6, the main mediators of the response, have been shown to be elevated in patients with type 2 diabetes and with the metabolic syndrome (2). It is well known that in the general population, as well as in diabetes, these acute-phase markers are associated with increased cardiovascular risk, because chronic inflammation is one of the pathogenetic mechanisms of atherosclerosis (3). In contrast, the relationships between low-grade inflammation and diabetic microangiopathy are still unclear. As far as nephropathy is concerned, several studies have examined the relationships with inflammation, leading to conflicting results (4–11). Overall, however, most studies have reported an increase in acute-phase markers in patients with nephropathy and also in patients with microalbuminuria (5,7–9). The coexistence of an inflammatory condition with diabetic nephropathy could explain in part the tremendously increased cardiovascular risk among these patients.

Also fibrinogen has been reported to be associated with both cardiovascular risk and nephropathy in type 1 and type 2

diabetes (10,11). The Diabetes Control and Complications Trial/Epidemiology of Diabetes Intervention and Complications (DCCT/EDIC) study group reported that fibrinogen is associated with AER, especially in men, but not with retinopathy and that a fibrinogen gene polymorphism is associated with peripheral vascular disease (11). It is interesting that hyperfibrinogenemia was associated with components of the insulin-resistance trait cluster (11). In type 2 diabetes, fibrinogen levels have been demonstrated to predict the progression to overt nephropathy (10). Hyperfibrinogenemia, an indicator of inflammation, is also associated with the presence of endothelial dysfunction, insulin resistance, hypercoagulability, and increased blood viscosity and is a marker of unstable atherosclerotic lesions (11–15). The aim of the present study was to explore the relationships between low-grade inflammation markers, glomerular structure, and renal function in patients with type 2 diabetes.

Materials and Methods

Patients

Seventy-four patients with type 2 diabetes were studied. Exclusion criteria were serum creatinine >180 $\mu\text{mol/L}$, presence of obvious non-diabetic renal diseases, and secondary causes of hypertension, including known renal artery stenosis and endocrinopathies. Patients were defined as microalbuminuric when albumin excretion rate (AER) was between 20 and 200 $\mu\text{g/min}$ in at least two of three sterile 24-h urine collections and as proteinuric when AER was >200 $\mu\text{g/min}$. Patients

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were defined as being hypertensive when BP values were >130/85 mmHg or when antihypertensive therapy was used (angiotensin-converting enzyme inhibitors or angiotensin receptors blockers alone or associated with dihydropyridine calcium antagonists and/or diuretics and α - and β -blockers). Patients were admitted to the Department of Medical and Surgical Sciences at the University of Padova, where baseline evaluation were performed (medical history, physical examination, renal functional studies, and kidney biopsy). All patients gave their written informed consent before the study. This study protocol was approved by the Ethics Committee of the University of Padova.

Methods

In all patients, venous blood samples were collected in the fasting state for determination of fibrinogen, leukocytes, and erythrocyte sedimentation rate (ESR); sera of 55 patients were available for SAA, CRP, and IL-6 determination.

AER was measured by an immunoturbidimetric method on three sterile 24-h urine collections. GFR was measured by modeling analysis of plasma decay of ^{51}Cr -EDTA as described elsewhere (16). The normal range in a group of 19 age and gender-matched normal control subjects was 85 to 135 ml/min per 1.73 m². BP was measured at least 10 times with the patients in the supine position, and the values provided the mean of these repeated measurements. HbA_{1c} was measured by HPLC (Variant II Analyzer; Bio-Rad, Hercules, CA).

Plasma fibrinogen levels were measured using a commercially available assay kit based on clotting method of Von Clauss. Serum SAA and CRP were measured by a particle-enhanced nephelometric immunoassay on a fully automated BN II nephelometer system (Dade Behring, Milan, Italy) (17). Both assays are based on an immune reaction involving specific antibodies, covalently coated with core shell-type particles. For SAA, the coefficient of variation ranges from 3.3 to 5.0% (within-run) and 3.1 to 6.9% (between-run). The reference limit is 6.8 mg/L. ESR was measured by the capillary photometric-kinetic technology on the Microtest 1 (SIRE Analytical System, Udine, Italy). Reference limits for men and women are as follows: from 15 to 50 yr of age, 28 and 38 mm, respectively; from 51 to 70 yr, 37 and 39 mm, respectively (18). Quantitative measurement of IL-6 was done by a solid-phase, enzyme-labeled, chemiluminescent sequential immunometric assay on a Immulite 1000 analyzer (Medical System, Genova, Italy). The coefficient of variation within and between run ranges from 5.1 to 7.5%.

A percutaneous kidney biopsy was performed under ultrasound guidance in all patients. Tissue was immediately processed for light, electron, and immunofluorescence microscopy. Electron microscopic examination was conducted on tissue that was fixed in 2.5% glutaraldehyde in Millonig buffer and fixed in Polybed (19). At least three glomeruli for each biopsy were examined. Glomeruli were photographed at a magnification of $\times 3900$ to produce photomontages of the entire glomerular profile. The montages were used to estimate mesangial fractional volume [Vv(mes/glom)] by point counting (normal values, 0.19 ± 0.03) (19). Another set of micrographs, obtained at $\times 12,000$ magnification, by systematically sampling approximately 20% of the glomerular profile, was used to estimate glomerular basement membrane (GBM) width by the orthogonal intercept method (19) (normal values, 320 ± 38 nm in 26 age- and gender-matched normal kidney donors). None of these patients had light, immunofluorescent, or electron microscopy findings of any definable renal disease other than diabetic nephropathy.

Statistical Analyses

Data are expressed as mean \pm 1 SD or as median and interquartile range. For checking the Gaussian distribution, data were preliminary

evaluated by applying the Kolmogorov-Smirnov test, taking $P < 0.001$ as significant. AER values, not normally distributed, were logarithmically transformed before analyses. Data were analyzed using the SPSS statistical package for Macintosh. One-way ANOVA was used to compare mean values among groups, followed by least significant difference tests for multiple comparisons. Univariate and multivariate regression analysis was used to evaluate the relationships between the variables studied. Values for $P < 0.05$ were considered significant.

Patients were divided into two groups on the basis of their GBM width: those with GBM width in the normal range (≤ 396 nm = mean + 2 SD in normal controls) and those above. Mann-Whitney U test and Kruskal-Wallis test were used to compare the results in the two groups.

We divided the distributions of variables into quintiles and used regression models to estimate the significance of trend in relative risk (RR) across increasing quintiles and to estimate risk for having GBM width above normal in each quintile relative to the lowest quintile. Relative risk is defined as the increased risk in one study group compared with the risk in another group. The formula to obtain relative risk is as follows: $RR = [TP/(TP+FN)]/[FP/(FP+TN)]$, where TP is true positive, FN is false negative, FP is false positive, and TN is true negative.

Results

Relationships between Renal Function and Inflammatory Markers

Twenty-three patients were normoalbuminuric (AER, 6 $\mu\text{g}/\text{min}$ [median, range, 2 to 18 $\mu\text{g}/\text{min}$]), 30 were microalbuminuric (AER, 76 $\mu\text{g}/\text{min}$ [range, 20 to 175 $\mu\text{g}/\text{min}$]), and 21 were proteinuric (AER, 646 $\mu\text{g}/\text{min}$ [range, 215 to 3958 $\mu\text{g}/\text{min}$]). Table 1 summarizes the clinical features of patients. Age, diabetes duration, GFR, and BP were similar in the three groups; in contrast, HbA_{1c} was higher in proteinuric than in normoalbuminuric patients. GBM width and Vv(mes/glom) increased from normoalbuminuric to microalbuminuric and proteinuric patients [$P < 0.005$ normoalbuminuric and microalbuminuric *versus* proteinuric for GBM width and $P < 0.01$ normoalbuminuric *versus* proteinuric for Vv(mes/glom)].

In Table 2, the inflammatory markers are summarized: ESR, CRP, fibrinogen, SAA, and IL-6 differed among groups, with proteinuric patients having the highest levels. In contrast, white blood cell count was not significantly different in the three groups.

AER was directly correlated to both GBM width ($r = 0.48$, $P < 0.001$) and Vv(mes/glom) ($r = 0.39$, $P < 0.005$); AER was also positively correlated with fibrinogen ($r = 0.47$, $P < 0.001$), SAA ($r = 0.28$, $P < 0.03$), and HbA_{1c} ($r = 0.26$, $P < 0.05$). When HbA_{1c} and inflammatory markers were included in a multiple regression model, only fibrinogen and HbA_{1c} explained AER ($r = 0.57$, $P < 0.02$, β 0.32 for fibrinogen; $P < 0.05$, β 0.24 for HbA_{1c}). GFR was not significantly correlated to any inflammation marker.

Relationships between Glomerular Structure and Inflammatory Markers

Patients were divided into two groups on the basis of their GBM width: those with GBM width in the normal range (≤ 396 nm) and those above. AER was significantly higher in patients with GBM above normal (99 $\mu\text{g}/\text{min}$ [14 to 518 $\mu\text{g}/\text{min}$] *versus* 37 $\mu\text{g}/\text{min}$ [12 to 104 $\mu\text{g}/\text{min}$]; $P < 0.05$), whereas GFR was not significantly different (92 *versus* 108 ml/min per 1.73 m²). Pa-

Table 1. Clinical features of patients with type 2 diabetes divided in three groups: normoalbuminuric, microalbuminuric, and proteinuric^a

	NA	MA	P	P Values
<i>n</i>	23	30	21	
Age (yr)	61 ± 6	57 ± 8	60 ± 7	
Diabetes duration (yr)	13 ± 8	12 ± 5	16 ± 6	
BMI (kg/m ²)	28 ± 4	30 ± 5	28 ± 5	
HbA _{1c} (%)	7.6 ± 1.7	8.2 ± 1.5	9.0 ± 1.8	<i>P</i> < 0.02 <i>P</i> versus NA
Mean BP (mmHg)	109 ± 9	108 ± 10	108 ± 9	
GFR (ml/min per 1.73 m ²)	97 ± 24	103 ± 30	92 ± 36	
Vv(mes/glom)	0.23 ± 0.06	0.25 ± 0.05	0.28 ± 0.06	<i>P</i> < 0.01 <i>P</i> versus NA
GBM width (nm)	414 ± 73	423 ± 97	532 ± 144	<i>P</i> < 0.001 <i>P</i> versus NA and MA

^aData are expressed as mean ± 1 SD. NA, normoalbuminuric; MA, microalbuminuric; P, proteinuric; BMI, body mass index; Vv(mes/glom), mesangial fractional volume; GBM, glomerular basement membrane.

Table 2. Acute-phase markers of inflammation in patients with type 2 diabetes divided in three groups: NA, MA, and P^a

	NA	MA	P	P Values
WBC (<i>n</i> × 10 ⁹ /L)	6.2 (5.1–9.3)	7.1 (5.6–13.0)	7.2 (4.7–10.8)	
ESR (mm/h)	10 (4.4–28.2)	18 (8–41)	22 (8.2–62.4)	<i>P</i> < 0.03 <i>P</i> versus NA and MA
CRP (mg/L)	3.1 (2.1–7.7)	3.4 (2.7–8.8)	5.1 (3.0–12.9)	<i>P</i> < 0.04 <i>P</i> versus NA
Fibrinogen (g/L)	3.1 (2.5–4.4)	3.1 (1.9–4.6)	4.3 (2.9–6.2)	<i>P</i> < 0.001 <i>P</i> versus MA and NA
SAA (mg/L)	2.7 (1.2–8.3)	4.3 (1.0–9.2)	6.0 (1.8–21.6)	<i>P</i> < 0.03 <i>P</i> versus NA
IL-6 (μg/L)	2.4 (1.9–8.3)	2.2 (1.8–5.7)	4.0 (2.1–8.2)	<i>P</i> < 0.03 <i>P</i> versus NA and MA

^aData are expressed as median (interquintile range). WBC, white blood cells; SR, sedimentation rate; CRP, C-reactive protein; SAA, serum amyloid A protein.

tients with GBM >396 nm, compared with patients with GBM in the normal range, had higher levels of CRP (4.6 mg/L [3.1 to 11.5 mg/L] versus 3.1 mg/L [3.0 to 5.6 mg/L]; *P* < 0.003), SAA (5.4 mg/L [1.5 to 12 mg/L] versus 2.3 mg/L [1.0 to 8.1 mg/L]; *P* < 0.004), and IL-6 (3.7 ng/L [2.0 to 8.0 ng/L] versus 2.0 ng/L [2.0 to 4.4 ng/L]; *P* < 0.0004). The RR to have abnormal GBM width was in the highest quintile 2.96 for IL-6, 2.97 for SAA, and 2 for CRP.

Linear regression analysis showed that GBM width was related to diabetes duration (*r* = 0.38, *P* < 0.002), fibrinogen (*r* = 0.33, *P* < 0.002), and IL-6 (*r* = 0.25, *P* < 0.05). Vv(mes/glom) was not significantly related to any acute-phase marker.

Discussion

This study describes an association between several acute-phase markers of inflammation and albumin excretion rate in patients with type 2 diabetes. In addition, a correlation between some of the inflammatory markers and the presence of a crucial lesion of diabetic glomerulopathy, GBM thickening, was observed.

Several reports have suggested associations between different inflammatory markers and the presence and severity of diabetic nephropathy, although with conflicting results (5,7–9). In patients with type 1 diabetes, fibrinogen has been consistently shown to be increased in patients with nephropathy

(4,11), whereas opposite findings have been reported on IL-6 and CRP (4,9). In type 2 diabetes, fibrinogen is associated with the presence of nephropathy in Japanese patients (20). The Casale Monferrato Study (10) demonstrated that fibrinogen is an independent predictor of progression to overt nephropathy in white patients with type 2 diabetes; these findings are in keeping with a previous report describing fibrinogen and CRP as predictive of the changes over time in AER (8). Also, SAA protein, increased in Japanese patients with type 2 diabetes, is significantly correlated with AER (21). At variance with previous studies, we have determined several acute-phase markers and observed that fibrinogen, IL-6, CRP, SAA, and SR are higher in patients with overt nephropathy compared with patients with normal AER, thus supporting the hypothesis of a link between inflammation and diabetic nephropathy.

The main finding of the present study is the association between a crucial lesion of diabetic glomerulopathy, GBM thickening, and acute-phase markers. We previously described heterogeneity in renal structure among patients with type 2 diabetes; indeed, only a subset of patients with microalbuminuria and proteinuria have diabetic glomerulopathy (22). In a large longitudinal study, we also described that the degree of diabetic glomerulopathy [GBM width and Vv(mes/glom)] is a strong predictor of progression of renal disease (23). Similarly, in patients with type 1 diabetes, GBM width in the baseline

biopsy was predictive of AER over time (24). Thus, the determination of CRP, SAA, IL-6, and fibrinogen plasma levels might help to identify type 2–diabetic patients with increased risk for progression toward ESRD. The nature of the relationships between GBM width and acute-phase markers is unknown: It can be hypothesized that low-grade inflammation might stimulate endothelial cells and podocytes toward an accumulation of extracellular matrix in the GBM. Alternatively, the deposition of extracellular matrix in excess may induce an acute-phase reaction. Given the cross-sectional design of this study, it is impossible to sort out whether inflammation plays a pathogenetic role in the development of diabetic nephropathy or increased inflammatory markers represent a response to renal injury.

Fibrinogen may be associated with GBM thickening not only via inflammatory mechanisms but also through endothelial damage, coagulant activity, and platelet activation (11–15). SAA is an acute-phase protein that has been investigated poorly in human physiology and pathology. Only one study to date has explored the relationships between SAA and nephropathy in diabetes (21); the authors speculated that the increase in SAA may be related to an activation of IL-6 and TGF- β . If this acute-phase protein was related to the TGF- β system, then this could explain the link with extracellular matrix accumulation in the GBM, given the central role of TGF- β in matrix synthesis and degradation in diabetes (25). However, the relationships between SAA and TGF- β are only speculative and have not been investigated yet. Finally, we found an association among IL-6, AER, and GBM thickening. Although the relationships between IL-6 plasma levels and diabetic nephropathy in type 2 diabetes have not been explored, Suzuki *et al.* (26) studied IL-6 mRNA in kidney biopsies of Japanese patients with diabetic nephropathy. By *in situ* hybridization, they found a relationship between severity of diabetic glomerulopathy (mesangial expansion by light microscopy) and expression of IL-6 mRNA in glomerular cells (mesangial cells and podocytes) (26). Thus, it is plausible that IL-6 affects extracellular matrix dynamics at mesangial cell and podocyte levels, contributing to both mesangial expansion and GBM thickening. In conclusion, this study demonstrates that low-grade inflammation is associated with nephropathy status and GBM thickening in type 2 diabetes and suggests that the measurements of acute-phase markers may help to identify patients who are at high risk for progression.

Acknowledgments

This study was supported by grants from the Italian Ministry of Health and Ministero dell' Istruzione dell' Università e della Ricerca (FIRB 2001 RBNE01KCX4).

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