Mannose-Binding Lectin as a Predictor of Microalbuminuria in Type 1 Diabetes

An Inception Cohort Study

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Inflammation and complement activation via the mannose-binding lectin (MBL) pathway have been suggested to play a role in the pathogenesis of diabetic microvascular complications. The association between the complement-activating protein MBL and the development of persistent microalbuminuria was evaluated in an inception cohort of 286 newly diagnosed type 1 diabetic patients consecutively admitted to the Steno Diabetes Center between 1 September 1979 and 31 August 1984. Serum MBL was measured with an immunofluorometric assay in 270 of the patients (159 men) after 3 years of diabetes duration. During the median (range) follow-up period of 18.0 (1.0–21.8) years, 75 patients subsequently progressed to persistent micro- or macroalbuminuria (urinary albumin excretion rate >30 mg/24 h). In patients with MBL levels above the median (1,597 μg/l), the cumulative incidence of persistent micro- or macroalbuminuria was 41% (CI 31–50) as compared with 26% (CI 17–34) in patients with MBL levels below the median (log-rank test, $P = 0.003$). In a Cox proportional hazard model with sex and age as fixed covariates, MBL was independently associated with later development of persistent micro- or macroalbuminuria (hazard ratio 1.21 [CI 1.02–1.42] per 1,000 μg/l increase in MBL; $P = 0.03$) after adjusting for possible confounders. In our study, high levels of MBL early in the course of type 1 diabetes was significantly associated with later development of persistent micro- or macroalbuminuria, suggesting that complement activation initiated by MBL may be involved in the pathogenesis of diabetic microvascular complications. ***Diabetes*** **54:1523–1527, 2005**

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CRP, C-reactive protein; MBL, mannose-binding lectin; UAER, urinary albumin excretion rate.

A substantial portion of type 1 diabetic patients develop diabetic nephropathy, whereas others seem to be protected from this complication. Persistent microalbuminuria (urinary albumin excretion rate [UAER] of 30–300 mg/24 h) is an established risk factor for the development of overt diabetic nephropathy, characterized by albuminuria >300 mg/24 h (1). Microalbuminuria may be regarded as an early marker of diabetic kidney disease, as early renal structural lesions can be detected at this stage (2). Genetic susceptibility, metabolic abnormalities, hemodynamic changes, upregulated growth factors, and cytokines may all play a part in the development of diabetic glomerulopathy, although the complex pathogenesis of diabetic nephropathy is not fully understood (1). It has been suggested that inflammation and complement activation are involved in the pathogenesis of diabetic microvascular complications (3–6). Mannose-binding lectin (MBL; also known as mannan-binding lectin) can activate the complement system independent of antibodies via MBL-associated serine proteases, thereby initiating the so-called MBL pathway of complement activation (7). Through this pathway, MBL plays an important role in the innate immune system. The level of MBL varies considerably among individuals, mainly because of frequently occurring polymorphisms within exon 1 as well as in the promoter region of the *MBL2* gene on chromosome 10 (8). High levels of MBL are known to protect against invading microorganisms (9,10) and in other situations may mediate detrimental inflammation through exaggerated complement activation (11,12). In a recent cross-sectional study, it was demonstrated that in normoalbuminuric type 1 diabetic patients, circulating MBL levels correlate positively with the UAER (4). Higher levels of MBL have been found to be associated with micro- and macrovascular complications in type 1 diabetic patients (5). However, no prospective studies have evaluated the association between MBL and subsequent development of microvascular complications. In the present study, MBL was measured 3 years after diabetes onset in a large inception cohort of newly diagnosed type 1 diabetic patients. These patients were followed for a median of 18 years to evaluate in a prospective design the association between baseline MBL concentrations and the development of persistent microalbuminuria. The change in MBL levels during 15 years of diabetes was also assessed.

TABLE 1
Baseline characteristics of 270 type 1 diabetic patients followed from onset of diabetes and divided into those who did and those who did not progress to microalbuminuria

	Nonprogressors	Progressors	*P*
n	195	75	—
Men	108 (55)	51 (68)	0.06
Height (cm)	172 ± 13	168 ± 15	0.051
Weight (kg)	60.4 ± 13.6	59.5 ± 16.5	0.66
Age (years)	27 ± 13	28 ± 17	0.75
Systolic blood pressure (mmHg)	122 ± 16	128 ± 19	0.006
Diastolic blood pressure (mmHg)	76 ± 10	80 ± 12	0.005
Urinary albumin excretion rate (mg/24 h)†	8 (5–13)	11 (8–17)	0.001
HbA_{1c} (%)	9.7 ± 2.2	10.1 ± 1.8	0.16
Serum cholesterol (mmol/l)	5.4 ± 1.5	5.6 ± 1.4	0.39
Serum creatinine (mmol/l)	80 ± 16	77 ± 15	0.21
Fasting plasma C-peptide (nmol/l)	0.172 ± 0.093	0.174 ± 0.082	0.90

Data are means ± SD, *n* (%), or median (interquartile range). Total cohort included 286 patients; 9 were excluded from analyses and 7 had no measurement of MBL performed.

RESEARCH DESIGN AND METHODS

All newly diagnosed type 1 diabetic patients consecutively admitted to the Steno Diabetes Center between 1 September 1979 and 31 August 1984 were included in an inception cohort comprising 286 patients. This cohort has been previously described in detail (13). Of the 286 patients, 9 were excluded from analyses due to other serious concomitant diseases and 7 had no measurement of MBL performed at baseline. Thus, 270 type 1 diabetic patients were eligible for analyses in the present study.

All patients attended the outpatient clinic of the Steno Diabetes Center every 3–4 months as part of their routine follow-up. Patients were treated by diabetologists and nurses according to set principles and guidelines, as previously described (13,14). No specific intervention was carried out. From 1 January 1980, HbA_{1c} was measured at each visit (14). The method used for measuring HbA_{1c} from venous blood samples has changed over the years; the predominate method has been high-performance liquid chromatography ion-exchange using a normal range of 4.1–6.4% (13). The patients had their 24-h UAER measured at least once a year. Until 1984, UAER was quantitated using automated immunotopical nephelometric analysis (15); from 1984 to 1990, using radioimmunoassay (sensitivity 0.5 mg/l, coefficient of variation [CV] 9%) (16); and from 1990, using enzyme immunoassay (sensitivity 1.1 mg/l, CV 8%) (17). A very close correlation between radial immunodiffusion and radioimmunoassay ($r = 0.98$) (18) and radioimmunoassay and enzyme immunoassay ($r = 0.99$) (17) was documented before changing the methods. From 1997, the DAKO Turbidimetric method was used to measure UAER. This method is closely correlated with enzyme immunoassay ($r = 0.99$) and has a CV of 5%. Persistent micro- and macroalbuminuria were defined as a UAER of 30–300 mg/24 h and >300 mg/24 h in at least two of three consecutive samples, respectively, with at least a 30% increase above baseline (13).

Serum MBL concentrations were measured using an in-house, time-resolved immunofluorometric assay with a lower detection level of 10 μg/l (19). In brief, microtiter wells were coated with mannan then incubated with samples diluted 200-fold. After the samples were washed, monoclonal anti-MBL antibody (131–1; Immunolex, Copenhagen, Denmark) labeled with europium using reagents from Wallac (Turku, Finland) was added. After samples were incubated and washed, the amount of bound, labeled antibody was assessed by time-resolved fluorometry (Delfia; Wallac). A number of control serum samples covering different MBL levels were included in all assays. The CVs obtained were 10% for a sample of 3,004 mg/l, 7.5% for a sample of 1,330 mg/l, 4.9% for a sample of 238 mg/l, and 23% for a sample of 66 mg/l. In healthy subjects, the median day-to-day variability in serum MBL concentrations expressed as the CV was 5.6% (20). MBL was measured twice: initially 3 years after diabetes onset in all subjects (i.e., after initial glycemic stabilization) and before the development of persistent microalbuminuria. In patients progressing to persistent microalbuminuria, MBL was measured again 1 year after the development of the disorder. In persistent normoalbuminuric patients, MBL was measured 15 years after diabetes onset.

Arterial blood pressure was measured at least once a year with a standard mercury sphygmomanometer and an appropriate cuff size. The measurements were performed with the patient in the sitting position after ~10 min rest. Smoking history was elicited via a questionnaire, and patients were classified as smokers if they smoked more than one cigarette per day. Retinopathy was assessed through dilated pupils and graded as absent, simplex, or proliferative (14).

The local ethics committee (Copenhagen, Denmark) approved the experimental design, and all patients gave written informed consent to participate in this study.

MBL genotyping. Of the 270 patients, 216 gave informed consent for genetic risk factors involved in the development of micro- and macroalbuminuria to be evaluated. As previously described, a real-time PCR technique was used to genotype for polymorphisms in the human *MBL* (*MBL2*) gene (5). These polymorphisms comprised two variations in the 5′ regulatory region at positions −550 (H/L) and −221 (X/Y), one in the 5′ untranslated sequence at position +4 (P/Q), and three structural mutations within exon 1 at codons 52, 54, and 57, also known as the D, B, and C variants, respectively. The presence of one of the three structural mutations within exon 1 (designated "O") significantly reduces circulating MBL. Of the promoter polymorphisms, only the X/Y polymorphism influences serum MBL, with the presence of the X promoter variant causing MBL levels similar to that found in individuals with the structural gene variants (5). Genotypes were divided into low (YO/YO, XA/YO, YA/YO, and XA/XA) and high (YA/YA and XA/YA) MBL genotypes.

Statistical analysis. MBL, UAER, and serum creatinine concentrations had a positively skewed distribution, and consequently values are given as medians with interquartile ranges. All other values are given as means ± SD. Comparisons between groups were performed using the Mann-Whitney *U* test for nonnormally distributed variables and the unpaired Student's *t* test for normally distributed variables. A χ^2 test was used for comparing groups of noncontinuous variables. The cumulative incidence of persistent microalbuminuria was calculated based on the entire follow-up period ending in 2000, with a life-table method taking into account differences in the follow-up intervals; this method makes proper allowances for those censored observations and uses information from all subjects during follow-up to the time to event or censoring. Groups were compared using the log-rank test. The Cox proportional hazards regression model was used to evaluate the relative contributions of covariates to the risk of developing persistent microalbuminuria, correcting for different lengths of follow-up. To evaluate MBL as a causal determinant of the development of persistent microalbuminuria, a Cox regression model was used, including variables that either previously had been shown to be associated with the level of MBL or were correlated with MBL in the present study. Statistical significance was assumed for $P < 0.05$. All statistical calculations were performed with SPSS for Windows (version 12.0; SPSS, Chicago, IL).

RESULTS

The 270 patients were followed for a median of 18.1 (1.0–21.5) years, with a total of 4,581 patient-years of follow-up. In all, 75 patients progressed to persistent microalbuminuria, resulting in a cumulative incidence of persistent microalbuminuria of 32.9% (CI 26.5–39.3). Of these, 24 patients progressed further to persistent macroalbuminuria, with a cumulative incidence of 13.7% (8.0–19.4). Baseline clinical characteristics of the diabetic patients are summarized in Table 1. The UAER remained <30 mg/24 h in 195 patients, who were then defined as

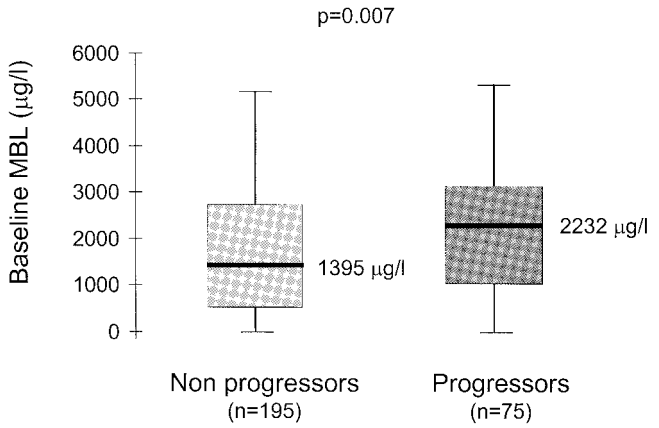

FIG. 1. Baseline circulating serum levels of MBL close to onset of type 1 diabetes in patients with persistent normoalbuminuria (nonprogressors; ▨) and patients later progressing to microalbuminuria (progressors; ■). Horizontal bars represent medians, columns indicates interquartile range, and vertical bars represent 95% CI. $P = 0.007$ by Mann-Whitney U test.

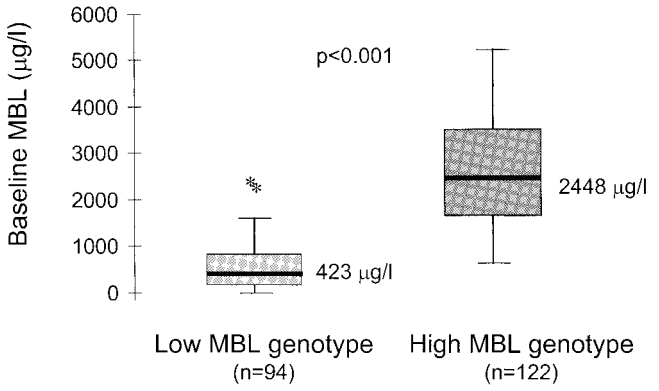

FIG. 2. Baseline circulating serum levels of MBL close to onset of type 1 diabetes in patients with low MBL genotypes (YO/YO, XA/YO, YA/YO, and XA/XA; ▨) and high MBL genotypes (YA/YA and XA/YA; ■). Horizontal bars represent medians, columns indicate interquartile range, and vertical bars represent 95% CI. $P < 0.001$ by Mann-Whitney U test. *Outlier.

nonprogressing subjects. At baseline, UAER and arterial blood pressure were significantly higher in patients who progressed to persistent microalbuminuria compared with those who did not (Table 1). Patients whose disease did progress tended to be male and have a lower height (Table 1). The two groups were similar with respect to age at diabetes onset, body weight, HbA_{1c}, serum cholesterol, creatinine, and fasting plasma C-peptide. No patients had diabetic retinopathy at baseline.

The median level of MBL 3 years after diabetes onset was significantly higher in patients later progressing to persistent microalbuminuria than in patients with persistent normoalbuminuria (2,232 [1,088–3,097] vs. 1,395 [492–2,731] μg/l; $P = 0.007$) (Fig. 1). There were no significant differences in circulating MBL levels between men and women ($P = 0.65$). Among the 216 patients genotyped for the MBL gene, the overall frequencies of the A/A, A/O, and O/O genotypes were 61, 33, and 6%, respectively, which was similar to the distribution of genotypes in healthy control subjects (56, 40, and 4%, respectively) (21). Circulating MBL levels were clearly reduced among heterozygous and homozygous carriers of exon 1 mutations as well as among patients homozygous for the X promoter variant; levels for low compared with high MBL genotypes were 423 (148–825) vs. 2,448 (1,704–3,447) μg/l ($P < 0.001$) (Fig. 2).

When the 270 patients were divided into those above and those below the median of serum MBL measured 3 years after diabetes onset (1,597 μg/l), the cumulative incidence of persistent micro- or macroalbuminuria was 41% (CI 31–50) in those above the median as compared with 26% (CI 17–34) in those below the median, with the hazard ratio for the development of microalbuminuria in patients above the median of MBL being 2.01 (CI 1.25–3.23; log-rank test: $P = 0.003$) (Fig. 3). In the 216 patients genotyped for the MBL gene, the hazard ratio for the development of microalbuminuria in those with a high MBL genotype was 1.42 (CI 0.86–2.36; log-rank test: $P = 0.17$).

Serum MBL correlated weakly with serum creatinine ($r = 0.15$, $P = 0.01$) and was associated with smoking status: MBL in smokers was 1,997 vs. 1,352 μg/l in nonsmokers ($P = 0.03$). No correlations between serum MBL and arterial blood pressure, HbA_{1c}, age at onset, height, serum cholesterol, or UAER at baseline were found.

In a Cox proportional hazards model with sex and age as fixed covariates, serum MBL measured 3 years after the onset of diabetes was significantly associated with later development of persistent micro- or macroalbuminuria (hazard ratio per 1,000 μg/l increase in serum MBL: 1.21 [CI 1.07–1.37]; $P = 0.003$). After adjusting for the confounding effects of smoking status, HbA_{1c}, blood pressure, and serum creatinine, MBL was independently associated with the development of micro- or macroalbuminuria (hazard ratio 1.21 [CI 1.02 –1.42] per 1,000 μg/l increase in serum MBL; $P = 0.03$). To compare the impact of MBL on the development of microalbuminuria, the association of mean arterial blood pressure and UAER with the development of microalbuminuria was 1.39 (CI 1.12–1.71) per 10-mmHg increase in blood pressure and 3.88 (CI 1.52–9.92) per 10-fold increase in UAER, respectively.

MBL was measured twice in 203 of the 270 type 1 diabetic patients. In 62 of the 75 patients whose disorder progressed, MBL was measured 3 years after diabetes onset and within 1 year after the development of persistent microalbuminuria. In these patients, MBL was measured after a mean follow-up time of 13.0 ± 4.7 years. In 141 of

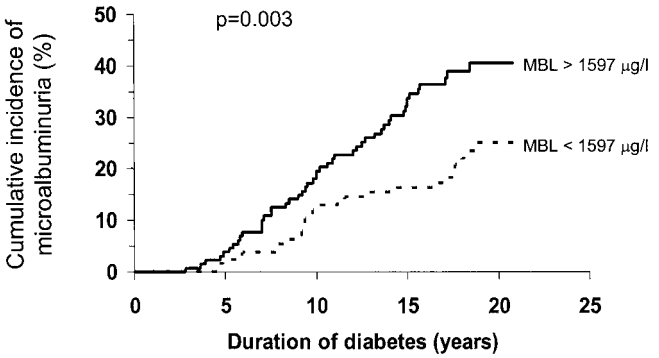

FIG. 3. Cumulative incidence of persistent microalbuminuria in 270 type 1 diabetic patients with onset of diabetes from 1979 to 1984, divided by the median level of MBL (1,597 μg/l) close to onset of type 1 diabetes. Hazard ratio for development of microalbuminuria in patients with MBL above the median: 2.01 (1.25–3.23). $P = 0.003$.

the 195 patients who did not progress to persistent microalbuminuria, MBL was measured 3 and 15 years after diabetes onset. In the second measurement of MBL, the median level of MBL was 1,131 (341–2,253) vs. 1,626 (785–2,532) μg/l in those who did not versus those who did progress to persistent microalbuminuria ($P = 0.04$). Among those who did develop microalbuminuria, the median change in MBL from baseline to 1 year after the development of microalbuminuria was −58 μg/l (−609 to 116) and among those who did not develop the disorder, the change in MBL from baseline to 15 years after onset of diabetes was −81 μg/l (−397 to 58) (NS). Age at diabetes onset was the only measured variable associated with a slight decrease in MBL over time ($r = 0.23$, $P = 0.001$).

DISCUSSION

In the present prospective, observational study of an inception cohort followed from the onset of type 1 diabetes for a median of 18 years, a cumulative incidence of persistent micro- or macroalbuminuria of 32.9% (CI 26.5-39.3) was found. When patients were divided according to the median level of MBL measured close to diabetes onset and before the development of microalbuminuria, there was a significantly higher cumulative incidence of persistent micro- or macroalbuminuria in patients with serum MBL above the median than in those patients with levels below the median (41% [CI 31–50] vs. 26% [17–34]; $P = 0.003$). Furthermore, we have demonstrated for the first time that a high level of MBL early in the course of type 1 diabetes is significantly associated with later development of persistent micro- or macroalbuminuria, suggesting that MBL-initiated complement activation may be involved in the pathogenesis of microvascular complications in diabetes.

MBL is a pattern-recognition molecule with the ability to recognize glycosylated surfaces. When it recognizes specific carbohydrate structures, MBL initiates the complement system via activation of associated serine proteases. A large variability in serum concentrations of MBL, which to a large extent are determined by mutations in the promoter region and within exon 1 of the *MBL2* gene on chromosome 10, has been found (8). There is growing evidence for a link between complement activation and the development and progression of renal disease (5,6,22,23), and it could be hypothesized that high levels of MBL may contribute to the development of diabetic microvascular complications through the MBL pathway of complement activation.

An association between MBL genotype, actual MBL levels, and diabetic nephropathy in type 1 diabetic patients has recently been reported (5). Because we genotyped only a subset of our patients and found a distribution of high and low genotypes similar to that previously reported (5,21), we divided our cohort into two groups according to the median level of serum MBL in the total cohort to minimize survival and selection bias. The median level of serum MBL coincided with the division between high and low MBL genotypes (5) (Fig. 2). Therefore, we could estimate the differences in the cumulative incidence of persistent micro- and macroalbuminuria between high and low MBL genotypes. Accordingly, our results support previous findings indicating that patients with high MBL genotype and therefore higher circulating MBL levels have a substantially higher risk of developing diabetic kidney disease (5). Using data from only patients who were genotyped reduced the number of patients in this subanalysis, making the confidence intervals wider.

Whether differences in circulating MBL levels are a contributing cause or a consequence of the development of microvascular complications due to diabetes cannot be established from cross-sectional studies. In the present prospective study, we found MBL levels to be elevated even before the development of microalbuminuria, and the association of MBL to the development of microalbuminuria persisted after adjusting for known confounders. Our results suggest that MBL may play a causal role in the development of microvascular complications in diabetes. Furthermore, the longitudinal analysis of MBL in our study showed a small decrease in MBL with increasing age and no difference between patients who progressed to microalbuminuria and patients with persistently normal UAE. This implies that rather than being a consequence of microvascular complications, MBL is more likely to be a contributing factor to the development of diabetic microangiopathy. However, whether MBL is a risk factor and thereby actually involved in the pathogenesis of diabetic nephropathy or merely a risk marker associated with other factors of importance for disease progression is not yet established.

In type 1 diabetes, C-reactive protein (CRP) levels have been found to be elevated and correlate with markers of endothelial dysfunction, suggesting that diabetic patients are in a state of chronic, low-grade inflammation (24). We did not measure CRP in the present study. MBL is an acute-phase reactant, although it reacts much slower than CRP. Previous studies in type 1 diabetic patients have found no correlation between MBL and CRP (5,25) or between MBL and the proinflammatory cytokine interleukin-6 (25). Furthermore, adjustments for differences in CRP levels have had no impact on the association between MBL and microvascular complications (5). Even though CRP and MBL are closely interrelated in inflammation and CRP may inhibit MBL activity (26), the evaluation of these markers of inflammation gives rise to different information in the diabetic patients. The association of blood pressure, endothelial damage, and serum MBL remains to be evaluated.

Oxidative stress leading to changes in cell surface glycosylations may activate the complement system via MBL (11). Assuming that ligands to MBL exist in renal tissue, this can in turn lead to renal MBL deposition, with deleterious effects of MBL causing local inflammation and fibrosis within the kidneys. The presence of MBL depositions in kidneys of diabetic patients has not yet been established; we evaluated only circulating MBL levels in our study as kidney biopsies were not available.

Diabetic kidney disease is strongly associated with cardiovascular mortality (27), and the Steno hypothesis, as proposed by Deckert et al. (28), advocates albuminuria to reflect a more generalized vascular process that involves the glomeruli in the kidneys, retina, and intima of large vessels simultaneously. MBL has been demonstrated to be elevated not only in patients with diabetic nephropathy, but also in diabetic patients with a history of cardiovascu-

lar disease irrespective of renal involvement (5). Emerging data indicate that complement activation via MBL may aggravate the effects of myocardial ischemia (29); furthermore, downstream inhibition of the complement system with a C5 inhibitor was recently shown to significantly reduce mortality after percutaneous coronary intervention in patients with myocardial infarction (30). The upregulation of MBL and activation of the complement system in diabetic patients may therefore be a candidate for a common link between micro- and macrovascular disease in these patients.

In conclusion, we have demonstrated for the first time that high levels of MBL early in the course of type 1 diabetes and before the development of microvascular complications is significantly associated with the later development of persistent micro- or macroalbuminuria. This suggests that MBL may be involved in the pathogenesis of microvascular complications in diabetes. Future research must assess whether intervention targeting the complement system in high-risk patients can alter the risk for the development of diabetic micro- and macroangiopathy.

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