Association Between Mannose-Binding Lectin and Vascular Complications in Type 1 Diabetes

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Complement activation and inflammation have been suggested in the pathogenesis of diabetic vascular lesions. We investigated serum mannose-binding lectin (MBL) levels and polymorphisms in the MBL gene in type 1 diabetic patients with and without diabetic nephropathy and associated macrovascular complications. Polymorphisms in the MBL gene and serum MBL levels were determined in 199 type 1 diabetic patients with overt nephropathy and 192 type 1 diabetic patients with persistent normoalbuminuria matched for age, sex, and duration of diabetes, as well as in 100 healthy control subjects. The frequencies of high- and low-expression MBL genotypes were similar in patients with type 1 diabetic and healthy control subjects. High MBL genotypes were significantly more frequent in diabetic patients with nephropathy than in the normoalbuminuric group, and the risk of having nephropathy given a high MBL genotype assessed by odds ratio (OR) was 1.52 (1.02–2.27, \( P = 0.04 \)). Median serum MBL concentrations were significantly higher in patients with nephropathy than in patients with normoalbuminuria: 2,306 μg/l (interquartile range [IQR] 753–4,867 μg/l) vs. 1,491 μg/l (577–2,944 μg/l), \( P = 0.0003 \). In addition, even when comparing patients with identical genotypes, serum MBL levels were higher in the nephropathy group than in the normoalbuminuric group. Patients with a history of cardiovascular disease had significantly elevated MBL levels independent of nephropathy status (3,178 μg/l [IQR 636–5,231 μg/l] vs. 1,741 μg/l [656–3,149 μg/l], \( P = 0.02 \)). The differences in MBL levels between patients with and without vascular complications were driven primarily by pronounced differences among carriers of high MBL genotypes (\( P < 0.0001 \)). Our findings suggest that MBL may be involved in the pathogenesis of micro- and macrovascular complications in type 1 diabetes, and that determination of MBL status might be used to identify patients at increased risk of developing these complications. *Diabetes* 53:1570–1576, 2004
positively with urinary albumin excretion (UAE) (15). The present study was performed to investigate the relationship among diabetic nephropathy, MBL gene polymorphisms, and circulating MBL concentrations in patients with type 1 diabetes. In addition, the relationship between MBL status and the prevalence of cardiovascular disease (CVD) was examined.

RESEARCH DESIGN AND METHODS

From the outpatient clinic at the Steno Diabetes Center, Gentofte, Denmark, all patients with long-standing type 1 diabetes and diabetic nephropathy whose glomerular filtration rate had been measured within the last year were recruited for a case-control study (16). A total of 199 patients with diabetic nephropathy and 192 patients with persistent normoalbuminuria (UAE <30 mg/24 h), matched for sex, age, and duration of diabetes, were included. Diabetic nephropathy was diagnosed clinically based on the following criteria: persistent albuminuria >300 mg/24 h in at least two of three consecutive 24-h urine collections, presence of retinopathy, and no evidence of other kidney or renal tract disease. A group of 100 age-matched healthy subjects with known MBL genotype (8) served as control subjects. All patients and control subjects were of Caucasian origin. The study was approved by the local ethics committee, and all participants gave their informed consent.

Assays. UAE was measured by enzyme immunoassay (15) from 24-h urine collections. HbA1c was measured by high-performance liquid chromatography (Diamat; Bio-Rad, Hercules, CA) with a normal range of 4.1–6.1%. Serum creatinine concentration was assessed using a kinetic Jaffe method. Serum MBL concentrations were measured by an in-house time-resolved immunofluorometric assay with a lower detection level of 10 μg/l (19). Numbers of control serum samples covering different MBL levels were included in all assays. The coefficient of variation (CV) obtained was 10% for a sample of 3,004 μg/l, 7.5% for a sample of 1,330 μg/l, 4.9% for a sample of 238 μg/l, and 23% for a sample of 66 μg/l. In healthy subjects the median day-to-day variability in serum MBL concentrations expressed as CV was 5.6% (20). A very sensitive in-house enzyme-linked immunosorbassay was used to measure highly sensitive C-reactive protein (hsCRP) with rabbit anti-CRP (Dako, Copenhagen) as a catching antibody and a horseradish peroxidase (HRP)-conjugated rabbit anti-CRP tagging antibody, with intra- and interassay CVs of 3.8 and 4.7%, respectively.

MBL genotyping. A newly developed real-time PCR technique was used to genotype for polymorphisms in the human MBL (MBL2) gene (21). These comprised two variations in the 5′ regulatory region at positions –550 (HL) and –221 (XY), one in the 5′ untranslated sequence at position +4 (PQ), and three structural mutations within exon 1 at codons 52, 54, and 57, also known as the D, B, and C variants, respectively. Three PCRs with two different conditions were sufficient to genotype one individual unambiguously. The three mutations in exon 1 were detected in one capillary using a sensor probe covering the codons, whereas amplification of the variants located upstream of the coding sequence was performed in only two reactions. Single-color detection was used for detection of the H/L polymorphism, and multiplexing by dual color probes was used for simultaneous genotyping of X/Y and P/Q.

The present study was performed in the morning after an overnight fast. Venous blood was drawn with minimal stasis from an antecubital vein. Clotted blood was centrifuged within 1 h and stored at –80°C. Lymphocytes were isolated from peripheral blood and DNA prepared using standard techniques. Arterial blood pressure (BP) was measured twice and averaged after at least 10 min of rest in the supine position. A 12-lead electrocardiogram was recorded and subsequently coded independently by two trained observers who were masked to the clinical status of the patients, using the Minnesota Rating Scale (17). Ischemic heart disease was diagnosed if the electrocardiogram showed signs of probable MI (Minnesota Rating Scale 1.1–1.2) or possible myocardial ischemia (Minnesota Rating Scale 1.3, 4.1–4.4, 5.1–5.3, or 7.1), or if patients reported a history of either angina pectoris or MI defined in accordance with Rose and Blackburn (17) and World Health Organization (WHO) criteria. Positive manifestations of CVD were signs of ischemic heart disease as defined above or a history of stroke or intermittent claudication when interviewed with the WHO cardiovascular questionnaire (17). Smokers were defined as individuals smoking more than one cigarette/cigar/pipe a day; all others were classified as nonsmokers.

RESULTS

Clinical characteristics of the diabetic patients are summarized in Table 1. Patients with nephropathy were well matched to patients with normoalbuminuria with regard to age, sex, and duration of diabetes. Patients in the nephropathy group had higher HbA1c, serum creatinine, total cholesterol, and systolic and diastolic BP levels, whereas BMI and smoking frequency were comparable in the two groups. Significantly more patients with than without nephropathy received antihypertensive treatment, whereas no patients were prescribed statins at the time of blood sampling. Positive manifestations of CVD were present in 84 patients, with a higher prevalence among patients with nephropathy compared with the normoalbuminuric group (30 vs. 12% [60 of 199 and 24 of 192, respectively], P < 0.0001). The mean age in the control group was identical to the patients (42.5 ± 10.3 years), and the sex-distribution ratio was 43 men and 57 women.

The distribution of all MBL genotypes with corresponding median serum MBL concentrations is shown in Table 2. The overall frequencies of A/A, A/O, and O/O genotypes among patients were 57, 39, and 4%, respectively, which was similar to the distribution of genotypes in healthy control subjects (56, 40, and 4%, respectively) (8). 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 (low MBL genotypes). The frequency of high MBL genotypes was significantly higher in patients with nephropathy than in the normoalbuminuric group (Table 3). The risk of having nephropathy given a high MBL genotype assessed by odds ratio (95% CI) was 1.52 (1.02–2.27). As shown in Fig. 1, circulating levels of MBL were significantly higher in patients with nephropathy than among patients with normoalbuminuria, and they were higher in both groups of type 1 diabetic patients than in healthy subjects. The difference in MBL concentrations between the nephropathy and normoalbuminuric groups (mean difference 1.458 μg/l [95% CI 0.887–2.029 μg/l]) remained statistically signifi-
TABLE 1
Clinical characteristics of 192 type 1 diabetic patients with normoalbuminuria and 199 type 1 diabetic patients with nephropathy

| Genotype | Number | MBL concentration (μg/l) |
|----------|--------|-------------------------|
| A/A      |        |                         |
| HYPA/HYPA| 29 (7.4)| 3,353 (1,447–12,598) |
| HYPA/LYPA| 11 (2.8)| 4,849 (1,151–14,945) |
| HYPA/LYQA| 42 (10.7)| 4,501 (1,031–16,699) |
| HYPA/LYPA| 49 (12.5)| 2,980 (806–16,089)  |
| HYPA/LYQA| 4 (1.0) | 3,389 (1,860–5,010)  |
| HYPA/LYPB| 12 (3.1)| 3,846 (2,619–7,282)  |
| LYPA/LXP| 10 (2.3) | 3,944 (1,118–7,325)  |
| LYPB/LYPB| 14 (3.6) | 4,463 (2,473–15,025) |
| LYPB/LYPB| 8 (2.0)  | 796 (366–1,247)     |
| LYPB/LYPB| 21 (5.4) | 519 (319–1,410)     |
| A/O      |        |                         |
| A/B      |        |                         |
| HYPA/LYPB| 44 (11.3)| 738 (184–1,960)       |
| LYPB/LYPB| 8 (2.0)  | 796 (366–1,247)      |
| LYPB/LYPB| 25 (6.4)| 519 (319–1,410)      |
| LYPB/LYPB| 25 (6.4)| 519 (319–1,410)      |
| A/C      |        |                         |
| LYPQ/LYQA| 1 (0.3) | 1,062                   |
| LYPQ/LYQA| 1 (0.3) | 488                    |
| LYPQ/LYQA| 2 (0.5) | 151 (92–209)          |
| A/D      |        |                         |
| HYPA/HYPD| 19 (4.9)| 1,987 (859–3,060)     |
| HYPA/HYPD| 2 (0.5) | 2,859 (1,544–4,173)  |
| LYPQ/HYPD| 10 (2.6)| 1,436 (707–3,238)     |
| LYPQ/HYPD| 10 (2.6)| 1,436 (707–3,238)     |
| LYPQ/HYPD| 10 (2.6)| 1,436 (707–3,238)     |
| O/O      |        |                         |
| B/B      |        |                         |
| LYPB/LYPB| 5 (1.3) | 134 (25–208)           |
| LYPB/LYPB| 2 (0.5) | 48 (10–76)             |
| LYPB/LYPB| 9 (2.3) | 67 (29–447)            |
| LYPB/LYPB| 2 (0.5) | 30 (18–41)             |

Data are n (%) or median (range).

The higher median MBL concentration in patients with nephropathy was not merely attributable to the higher frequency of high MBL genotypes. Even when comparing patients with identical genotypes, median serum MBL concentrations were higher in patients with nephropathy than in patients with normoalbuminuria. As depicted in Fig. 2, the differences in serum MBL were only seen among patients with high MBL genotypes (4.370 μg/l [IQR 2,652–6,365] vs. 2.946 μg/l [2,114–4,036] in the nephropathy vs. normoalbuminuric groups, respectively; P < 0.00001) and not among low MBL genotypes (612 μg/l [IQR 205–1,113] vs. 801 μg/l [277–990] in the nephropathy vs. normoalbuminuric groups, respectively; P = 0.90). Serum concentrations of CRP were significantly higher among patients with than without nephropathy (1.25 mg/l [IQR 0.56–3.06] vs. 0.89 mg/l [0.33–2.06], P = 0.002), whereas serum CRP was unaffected by MBL genotype (1.09 mg/l [IQR 0.44–2.43] vs. 1.07 mg/l [0.47–2.75] in patients with high and low MBL genotypes, respectively; P = 0.82). There were no correlations between hsCRP and MBL levels when all patients were considered (r = 0.08, P = 0.12) or when the nephropathy and normoalbuminuric groups were analyzed separately (r = 0.13, P = 0.08; and r = –0.02, P = 0.75, respectively).

There were no significant sex differences in circulating MBL levels (1,600 μg/l [IQR 726–3,183] vs. 2,096 μg/l [624–4,030] in women vs. men, respectively; P = 0.41), nor in the distribution of high and low MBL genotypes (high-to-low ratio 54:46 vs. 53:47 in women vs. men, respectively; P = 0.40). MBL levels were not significantly correlated to age, duration of diabetes, or daily insulin dose, whereas there was a significant, albeit weak, positive correlation between MBL concentrations and HbA1c in the entire study group (r = 0.17, P = 0.001). This correlation was considerably stronger when only patients with high MBL genotypes were considered (r = 0.35, P < 0.00001, n =
TABLE 3
Distribution of low and high MBL genotypes in 391 type 1 diabetic patients with normoalbuminuria or nephropathy

|                 | Normoalbuminuria | Nephropathy | Healthy subjects |
|-----------------|------------------|-------------|------------------|
| n Low MBL genotypes | 192              | 199         | 100              |
|                 | 100 (52.1)       | 83 (41.7)   | 52 (52)          |
| High MBL genotypes | 92 (47.9)       | 116 (58.3) | 48 (48)          |

Data are n (%).

208) and was present in both the nephropathy and normoalbuminuric groups (r = 0.30, P = 0.001, n = 116; and r = 0.21, P = 0.048, n = 92; respectively). There were weak positive correlations between MBL and both total cholesterol (r = 0.19, P = 0.0001) and systolic BP (r = 0.13, P = 0.01), but MBL concentrations did not differ between patients with or without prescribed antihypertensive treatment (1,680 µg/l [IQR 677–3,791] vs. 1,447 µg/l [580–2,936], respectively, in normoalbuminuric patients [P = 0.58]; and 2,448 µg/l [IQR 723–4,900] vs. 1,998 µg/l [770–4,888], respectively, in patients with nephropathy [P = 0.85]). MBL levels did not correlate with serum creatinine when the nephropathy and normoalbuminuric groups were analyzed separately.

MBL concentrations were significantly higher among patients with positive manifestations of CVD than among patients without CVD (mean difference 880 µl [95% CI 169–1,591]) (Fig. 3A), and this difference remained statistically significant after correction for differences in hsCRP (mean difference 869 µl [155–1,582]). However, the distribution of high and low MBL genotypes was not significantly different between the two groups (high-to-low ratio 58:42 vs. 52:48 in patients with vs. without CVD, respectively; P = 0.17). When patients with high and low MBL genotypes were considered separately, the difference in MBL levels between patients with and without CVD was most pronounced among patients with high MBL genotypes (Fig. 3B and C). As mentioned above, positive manifestations of CVD were more frequent in patients with than without nephropathy, but even in patients with normoalbuminuria and high MBL genotypes, MBL concentrations were significantly higher among patients with CVD than among patients with no manifestations of CVD (4,468 µg/l [IQR 3,553–4,553] vs. 2,773 µg/l [2,085–3,723], P = 0.002). CRP levels were also significantly higher among patients with than without a history of CVD (1.45 mg/l [IQR 0.65–3.21] vs. 1.03 mg/l [0.42–2.41], P = 0.011), but when patients with normoalbuminuria and nephropathy were considered separately, the difference was only significant in the normoalbuminuric group (2.07 mg/l [IQR 0.89–4.18] vs. 0.79 mg/l [0.31–1.71], P = 0.0004; and 1.35 mg/l [0.52–2.89] vs. 1.20 mg/l [0.61–3.09], P = 0.85; in patients with normoalbuminuria and nephropathy, respectively).

**DISCUSSION**

The presented results suggest a role of MBL and the lectin pathway of complement activation in the pathogenesis of vascular complications in type 1 diabetes. High-expression MBL genotypes were more frequent among patients with diabetic nephropathy than among patients with normoalbuminuria, and even when comparing subjects with identical high MBL genotypes, circulating MBL levels were significantly higher in the nephropathy group than in the normoalbuminuric group independent of serum creatinine concentrations. Furthermore, type 1 diabetic patients with a history of CVD had significantly elevated levels of MBL independent of nephropathy status.

The median MBL concentration among healthy Caucasians is ~800–1,000 µg/l (7,8), but as a consequence of the frequently occurring polymorphisms in the promoter region and within exon 1 of the MBL2 gene, approximately one-third of the population has MBL concentrations <500 µg/l and >10% have concentrations <50 µg/l (8). Within-subject variations in MBL concentrations over time are small (20), and although serum levels may increase two- to threefold during an acute-phase response (7), the major part of between-subject variations in MBL concentrations remains genetically determined (23). The difference in circulating MBL concentrations between patients with type 1 diabetes and healthy subjects found in the present...
study extends our previous finding in patients with uncomplicated type 1 diabetes (15). The higher concentrations of
MBL in diabetic subjects was not explained by genetic
differences, because we, in line with a recent Japanese study
(24), found identical frequencies of the different genotypes in
patients and healthy control subjects. A plausible explana-
tion for the differences in MBL levels is hepatic portal
hypoinsulinemia, which generally occurs in type 1 diabetic
patients treated with subcutaneous injections of insulin. We
have recently demonstrated a significant suppressive effect
of insulin on circulating MBL levels in critically ill patients
(7). MBL is synthesized exclusively in the liver, and it seems
conceivable that hepatic MBL expression may be chronically
upregulated in type 1 diabetic patients as a consequence of
low portal insulin concentrations.

The distinct difference in MBL levels between diabetic
patients with nephropathy and patients with normoalbu-
munuria was in part attributable to differences in the MBL
genotype distribution. High-expression MBL genotypes
occurred with increased frequency among patients with
nephropathy, and although the odds ratio was relatively
small, this seems to indicate that inherited high concen-
trations of circulating MBL may be a risk factor for
diabetic nephropathy. Mounting evidence suggests that
there may be a link between complement activation and
the development of diabetic renal complications (25,26),
and MBL-mediated complement activation has recently
been implicated in the pathogenesis of other renal dis-
eases, such as IgA nephropathy and Henoch-Schonlein
purpura nephritis (27,28). It could thus be hypothesized
that in diabetic patients, high levels of MBL may contrib-
ute to the development of nephropathy through aggra-
vated complement activation.

In addition to the genetically determined differences, we
found that serum MBL concentrations were higher in
patients with than without nephropathy, even when com-
paring subjects with identical genotypes. Elevated levels of
MBL have been reported in a single study of patients
with chronic renal failure of unspecified causes (29). In
that study there was no relationship between serum MBL
levels and glomerular filtration rate, and because MBL is
not excreted or degraded by the kidneys (30), it is unlikely
that differences in MBL levels between the nephropathy
and normoalbuminuric groups are caused by differences in
renal function. It has been suggested that patients with
type 1 diabetes are characterized by a state of chronic
low-grade inflammation (31). A well-known feature of this
inflammatory activity is increased concentrations of acute-
phase proteins such as CRP, which has been shown to
predict cardiovascular mortality in both diabetic and non-
diabetic subjects (32). MBL is a slower-reacting and much
weaker acute-phase reactant than CRP (7), but it is
possible that the differences in MBL concentrations be-
tween patients with and without nephropathy may reflect
differences in inflammatory activity. We did indeed ob-
serve significantly higher hsCRP levels among patients
with nephropathy compared with patients with normo-
albuminuria. However, the differences in MBL levels
between the groups remained statistically significant after
correction for differences in hsCRP, which indicates that
CRP and MBL may carry different types of information as
markers of inflammation. Measurements of MBL levels, at
least among carriers of high MBL genotypes, may thus turn
out to be a supplement to hsCRP measurements in the
assessment of ongoing low-grade inflammation.

Most studies of the relationship among disease, MBL
contentations, and gene polymorphisms have focused on the
beneficial anti-infectious characteristics of MBL, and it
has been suggested that low MBL genotypes may predis-
pose to accelerated development of atherosclerosis.
through an increased risk of inflammatory infections (33,34). From an evolutionary point of view, however, the high prevalence of mutations in the MBL gene suggests that low levels of MBL in some situations may be advantageous (9). Under normal circumstances circulating MBL does not react with the host’s own tissues (35), but changes in cell surface glycosylations after cellular hypoxia may lead to increased MBL deposition and complement activation (36). MBL has been shown to aggravate the resulting ischemic injury in a rat model of acute MI (14), and new data suggest that complement activation after the ischemia and reperfusion during thoracoabdominal aortic aneurysm repair is MBL mediated (12). In a recent study, downstream inhibition of the complement system with a C5 inhibitor significantly reduced mortality after percutaneous coronary intervention in patients with MI (37). It is well established that diabetic patients have an adverse prognosis after percutaneous coronary intervention for acute MI in comparison with nondiabetic subjects (38), and it could be hypothesized that increased levels of MBL may contribute to this difference through increased activation of the complement cascade.

One of the important mechanisms responsible for the increased frequency of cardiovascular complications in diabetes is the nonenzymatic reaction between glucose and proteins or lipoproteins in arterial walls. Glucose forms reversible early-glycosylation products with reactive amino groups (Schiff bases), which subsequently rearrange to form the more stable Amadori-type early glycosylation products (e.g., HbA\textsubscript{1c}), which then again may form advanced glycosylation end products (39). Advanced glycosylation end products are known to accelerate the atherosclerotic process through a number of different mechanisms (40), but whether these nonenzymatic glycosylations may alter the autoreactivity of MBL remains uncharted. We found significantly higher concentrations of MBL in patients with than without a history of CVD. As expected, positive manifestations of CVD were more frequent among patients with nephropathy, but the association between high MBL and these vascular complications was present irrespective of renal status. This finding could suggest a possible role of MBL in the pathogenesis of macrovascular complications, but further prospective studies are needed to elucidate whether MBL is in fact a cause of or merely a marker for CVD in type 1 diabetes.

In conclusion, our study suggests that determination of the MBL status in patients with type 1 diabetes may prove beneficial to identify those at enhanced risk of developing micro- and macrovascular complications. Such screening should preferably include both serum MBL measurements and genotyping. The identification of patients at high risk would allow for increased vigilance, but further studies are needed to determine whether specific inhibition of MBL and the lectin pathway of complement activation may be a therapeutic option.
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