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Innate immune functions in kidney transplantation

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Chapter 3

Association between Mannose-binding lectin levels and graft survival in kidney transplantation

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Summary

The mannose-binding lectin (MBL) pathway of complement is activated by pattern recognition. Genetic MBL variants are frequent and are associated with low MBL serum levels. We hypothesized that higher MBL levels may be associated with more complement-mediated damage resulting in inferior graft survival.

Pretransplant serum samples collected from 266 consecutive deceased donor kidney transplant recipients were analyzed for MBL concentration by ELISA. Subsequently the cohort was analyzed for transplant-related outcome.

There was no significant difference in the incidence of delayed graft function in recipients with a low MBL level (≤ 400 ng/ml) compared to those with a higher MBL level (> 400 ng/ml) (37.1 vs. 34.9%). At 10-years, the death censored graft survival was 89.9% in patients with an MBL level below 400 ng/ml compared with 78.8% in patients with a higher MBL level ($P < 0.02$). Multivariate analysis including traditional risk factors for graft loss showed an independent risk of 2.7 (95% CI 1.2-6.3) for death censored graft loss if pretransplant MBL levels were above 400 ng/ml. This difference was almost entirely explained by rejection-associated graft loss (2.4 vs. 12,4%, $P < 0,01$).

In our cohort higher MBL levels seem to be associated with a more severe form of rejection leading to treatment failure and graft loss. If these data can be confirmed pretransplant MBL levels may provide additional information for risk stratification prior to kidney transplantation.

Introduction

Recently the interest in the role of the innate immune system in organ transplantation has increased. Within the innate immune system complement is thought to be one of the major inflammatory mediators particularly in the setting of ischemia/reperfusion injury [1;2]. In a mouse model of acute kidney rejection, disruption of the gene encoding for the complement component C3 in the transplanted kidney led to marked improvement of organ survival. In human transplantation a role for complement activation has been established by showing the presence of the complement split product C4d as a marker of acute humoral rejection [3;4] and its association with chronic transplant glomerulopathy [5].

Next to activation of the complement system via the classical or alternative pathway, the lectin complement pathway may play a role in renal transplantation. The collectin mannose-binding lectin (MBL) binds via its carbohydrate recognition domain to saccharides such as D-mannose, L-fucose and N-acetylglucosamine [6] on various microorganisms. This interaction leads to the activation of the MBL-associated serine proteases (MASP) and cleavage of C4 and C2 followed by formation of the C3 convertase C4b2a. In addition to activating the lectin complement pathway, MBL can mediate phagocytosis of opsonized organisms.

The serum MBL concentration shows a large inter-individual variation due to common mutations in the structural as well as the regulatory part of the MBL gene. Several studies have related MBL deficiency with an increased rate of infection in early childhood [7] and other conditions characterized by disturbed host defense [8;9]. Experimental data have shown that the lectin complement pathway contributes to activation of the complement cascade in the context of ischemia/reperfusion damage. Endothelial cells exposed to oxidative stress activate the lectin complement pathway *in vitro* [10]. A recent publication indicates that MBL also binds to both late apoptotic and necrotic cells [11]. *In vivo* studies show that inhibition of MBL with monoclonal antibodies leads to reduction of damage in a rat model of cardiac ischemia/reperfusion injury [12].

We hypothesize that MBL binding to injured tissue may lead to additional inflammation, thereby aggravating tissue damage and potentiating antigen presentation. Based on this hypothesis and the recent findings concerning the role of complement in ischemia/reperfusion damage and rejection we questioned whether higher recipient MBL levels might be associated with inferior outcome in the setting of kidney transplantation.

Methods

Study population

Pretransplant sera of 266 consecutive deceased donor kidney transplant recipients routinely collected at our institution from January 1990 to December 1994 were utilized for this study. Thirty-one recipients of the total number of kidney transplants performed during this period were excluded due to missing serum samples. Pretransplantation sera were routinely collected since 1989 and stored at –80°C. They had not been subjected to freeze/thaw cycles before analysis in our study. Sera from MBL-genotyped healthy controls (n = 70) [13] were used for comparison.

Data analysis was done using the Leiden Kidney Transplantation Database. This database contains donor variables (gender, age at time of death), recipient variables (age at time of transplantation, gender, panel reactive antibodies, CMV status), transplantation related factors (human leukocyte antigen-A [HLA-A], -B, and –DR mismatches; cold ischemia time; warm ischemia time), and post-transplantation features including immunosuppressive regimen, delayed graft function, rejection history, rejection treatment, dipstick proteinuria and serum creatinine values. Graft histology was evaluated retrospectively according to the Banff '97 classification [14]. After transplantation patients were followed until death, reinitiation of dialysis or December 2002. Delayed graft function was defined as the need for dialysis for more than 7 days post transplantation. Rejection-associated graft loss was defined as histologically proven acute rejection with ongoing functional deterioration despite antithymocyte treatment or chronic rejection leading to the reinitiation of dialysis treatment. Acute rejection episodes were treated according to a standard protocol consisting of methylprednisolone 1 g intravenously for three consecutive days; a 10d course of antithymocyte globulin at a dose 5mg/kg guided by absolute lymphocyte counts; or again methylprednisolone for the first, second (or steroid-resistant), or third rejection episode, respectively. An MBL concentration of 400 ng/ml was chosen as a cut-off to define individuals with normal and low MBL levels respectively. The higher and lower MBL groups were analyzed for differences in known predictors of transplantation outcome, such as the incidence of delayed graft function or acute rejection. Patients who lost their grafts within 3 months after transplantation were excluded from analysis for patient and graft survival.

ELISA

Serum MBL levels were assessed by sandwich ELISA as described previously [15]. In short 96-well ELISA plates (Greiner, Germany) were coated with 3E7 (mouse IgG1 anti-MBL at 2.5 µg/ml). After blocking residual binding sites with PBS containing 1% BSA and washing, serum samples were diluted 1/50 and 1/250 and incubated. Dig-conjugated 3E7 was added as detecting antibody. After washing detection of binding of Dig-conjugated antibodies was performed using HRP-conjugated rabbit anti-Dig Abs (Fab, from Boehringer Mannheim). Enzyme activity was detected using 2,2'-azino-bis(3-ethybenzthiazoline-6-sufonic acid)(Sigma). The optical density (OD at 415nm) was measured using a microplate biokinetics reader (EL312e; Biotek Instruments, Winooski, VT). A calibration line was produced using human serum from a healthy donor with a known concentration of MBL.

Statistical analysis

Categorical characteristics among MBL-groups were compared using cross-tables with calculation of the exact p-values. Continuous variables were analyzed using the Student t-test, when test assumptions were met, and otherwise with the Mann-Whitney test. Patient and graft survival was estimated using the Kaplan-Meier product-limit method and the curves were compared with the Log-Rank test. For analysis of differences in survival among MBL-groups, at individual time points, z-scores were calculated and p-values estimated using the standard normal distribution (Z-test).

To identify risk factors for graft loss and to adjust for potential confounding factors Cox Proprotional Hazards Regression was used.

P-values < 0.05 were considered to be statistically significant. All analyses were performed with SPSS Statistical Software Package (Version 10.07; SPSS, Inc., Chicago, Ill.).

Results

Follow up data were available for all transplanted patients. The mean MBL concentration of the 266 available sera was 1112 ng/ml (median 691 ng/ml; IQR 270-1697). These results were very similar to the levels we measured in healthy donors with a mean MBL level of 1054 ng/ml (median 679 ng/ml) [13]. The distributions of both the transplant recipients and the healthy donors are shown in figure 1. For analysis the patients were divided into groups with MBL levels below 400 ng/ml and

above 400 ng/ml. Using this cut-off 97 kidney recipients (36.5%) had a low MBL-level, which is comparable to our group of healthy donors (35.7% below 400 ng/ml) and to the frequency of variant alleles as defined in other populations [16]. In our genotyped control population 75% of those with an MBL level below 400 ng/ml have a variant MBL genotype (A/O or O/O) whereas 89% of those with an MBL level above 400 ng/ml have the wildtype MBL genotype (A/A) [13], showing a close association between MBL variant alleles and MBL levels below 400 ng/ml (P = 0.0001).

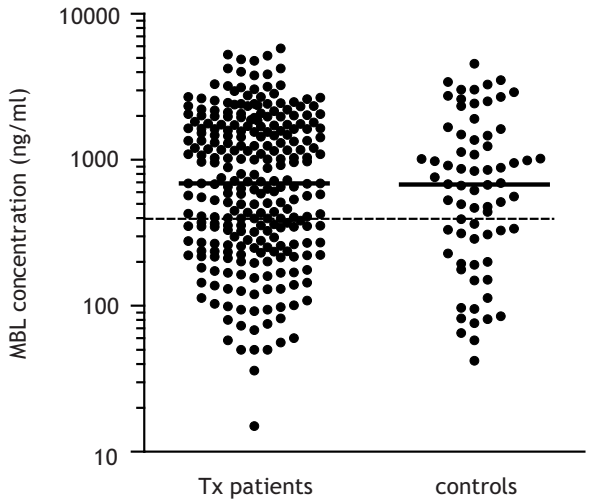

Figure 1. MBL concentration in pre-transplant sera and healthy controls. Horizontal solid lines indicate the median. The dashed line indicates the cutoff level used in the present study (400 ng/ml).

Between the two MBL groups there were no significant differences in recipient or donor age, years on dialysis, cold ischemia time, CMV serotype or sex distribution (Table 1). There was no difference in the distribution of the dialysis modality prior to transplantation.

The normal and low MBL groups were also compared for transplantation outcome. No significant difference in the incidence of delayed graft function (37.1% vs. 34.9%) or the incidence of first acute rejection episodes was found between the groups, illustrated in figure 2. Equally there was no difference if vascular and interstitial rejection or severity of rejection were analyzed separately (data not shown).

Table 1. Characteristics of study population according to MBL levels [a]

	Acceptor MBL-level (ng/ml)		
	MBL < 400	**MBL ≥400**	**P-value**
n	82	153	
Recipient age (yrs)	45	46.51	0.40
Donor age (yrs)	40.05	37.37	0.20
Hemodialysis (%)	48.1	48.3	0.98
Years on dialysis	4.78	3.8	0.09
CIT (h)	28.28	29.44	0.54
CMV sero-positive	59.3	48.4	0.11
Female (%)	41.5	32	0.10

[a] MBL, mannose-binding lectin; CIT, cold ischemia time; CMV, cytomegalovirus

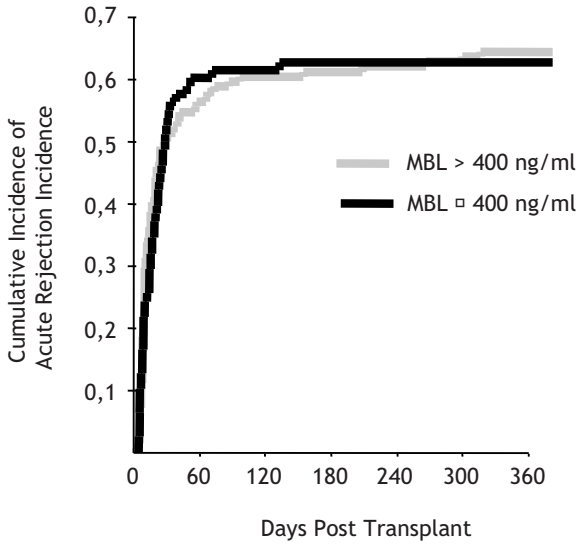

Figure 2. Cumulative incidence of first acute rejection according to MBL level.

For survival analysis all grafts that functioned for less than 3 months were excluded. This was done to exclude graft loss due to technical complications. At 3 months mean creatinine levels were the same in both the higher and lower MBL groups (168.8 µmol/l vs. 166.6 µmol/l, P = 0.82). There was a non-significant tendency towards

better patient survival in the group with a lower MBL level (P = 0.103) (figure 3). However overall graft survival was superior in acceptors with a low MBL level as shown in figure 4A (log-rank, P = 0.017). A similar difference was found when graft survival was censored for patient death with a functioning graft (log-rank, P = 0.028), (figure 3B). The 5-year death censored graft survival was 93.3% and 87.3% in the lower and the higher MBL group, respectively (P = 0.067). The 10-year death censored graft survival was 89.8% in the lower MBL group and 78.8% in the higher MBL group (P = 0.018).

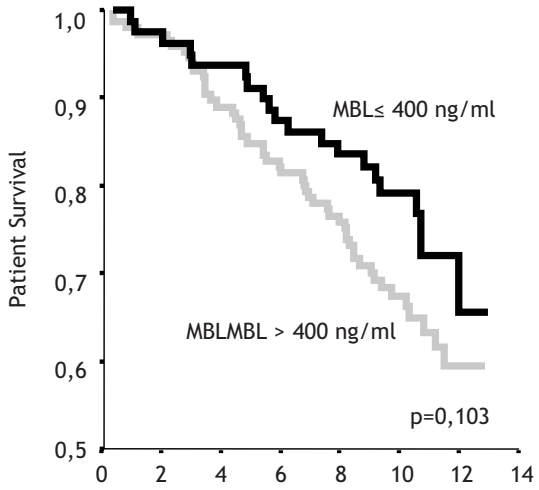

Figure 3. Patient survival according to MBL level.

As shown in table 2 the risk for death censored graft loss was significantly increased if the donor age was above 50 years (RR = 2.08; 95% CI, 1.1-4.04) or if acute rejection occurred (RR 4.18, 95% CI, 1.6-10.8). Neither HLA mismatches, a negative acceptor CMV serology nor cold ischemia time were significant risk factors for graft loss in our well-matched cohort. An MBL level above 400 ng/ml was associated with a 2.5 fold relative risk for death-censored graft loss (95% CI 1.1-1.57). When adjusted for other risk factors in a multivariate model, an MBL level above 400 ng/ml was shown to be an independent risk factor for graft loss. If the pre-transplantation MBL level was entered as a continuous parameter every 100 ng/ml concentration increase was associated with a RR of 1.03 (95% CI, 1.001-1.052). In the multivariate model the MBL level also proved to be an independent risk factor if analyzed as a continuous parameter (P = 0.009).

Table 2. Risk factors of death censored graft loss[a]

	Univariate		Multivariate	
	RR	**95% CI**	**RR**	**95% CI**
MBL> 400 ng/ml	2.50	1.1-5.7	2.76	1.2-6.32
Donor age >50 yr	2.08	1.1-4.04	2.23	1.14-4.3
Acute Rejection	4.18	1.6-10.8	4.26	1.64-11.00
CIT	1.03	0.98-1.08		
Acceptor CMV neg.	0.34	0.6-2.49		
HLA A-B ≥ 1mm	1.124	0.82-1.53		
HLA DR ≥1 mm	1.90	0.96-3.8		

[a] RR, relative risk; CI, confidence interval; CIT, cold ischemia time; HLA, human leukocyte antigen; mm, mismatch

To study the mechanism underlying MBL associated graft loss, we analyzed the reasons for graft loss in the low and high MBL groups (table 3). The excess graft loss in patients with a MBL above 400 ng/ml was almost entirely explained by an increased incidence of rejection associated graft loss (P = 0.01).

There was no difference in the need of a first or second treatment for acute rejection between both groups, whereas 22.9% of the kidney recipients with a MBL-level above 400 ng/ml did not adequately respond to antithymocyte treatment and thus recieved 3 or more courses of rejection treatment compared with 12.2% of the recipients with an MBL-level below 400 ng/ml (P = 0.03). (Data not shown)

Table 3. Reason for graft loss later than 3 months after transplantation according to MBL levels [a]

	Acceptor MBL-level (ng/ml)		
	MBL < 400	**MBL ≥ 400**	**P-value**
n	82	153	
No graft loss	58	84	0.02
Rejection associated graft loss	2	19	0.01
Recurrent disease	2	4	0.98
Death with functioning graft	17	40	0.42
Other	3	5	0.97

[a] MBL, mannose-binding lectin

Discussion

In the present study we analyzed the interaction between pre-transplantation MBL levels and outcome after deceased donor kidney transplantation. This is the first report showing that higher MBL levels are significantly associated with increased overall and death-censored graft loss. This difference was almost entirely explained by rejection-associated graft loss and coincided with a greater need for additional treatment for acute rejection after a course of antithymocyte globulin in the group with higher MBL-levels. The relative risk for graft loss with a pre-transplantation MBL levels above 400 ng/ml was comparable with the effect of receiving a graft from a donor over 50 years of age. Higher MBL levels were not associated with an increased incidence of delayed graft function or first acute rejection episodes.

The serum MBL concentrations in our study population were comparable with the levels found in other populations [13;17;18]. The inter-individual differences in MBL levels are largely explained by polymorphisms within the promoter region and in exon 1 of the *MBL2* gene. As a consequence the variation of MBL levels within individuals are small. Levels are only increased two to three-fold during acute phase responses in the critically ill and changes in MBL levels do not correlate with changes in C-reactive protein (CRP) during treatment on an intensive care unit [17]. In addition strong acute phase reactions in our transplant recipients are very unlikely since patients with evidence of acute infections or active inflammatory disease are not accepted for transplantation. With this in mind, it can be assumed that the MBL levels measured in pretransplantation samples reliably represent the mean MBL level in these individuals.

A single study has evaluated MBL levels in patients with advanced renal failure and hemodialysis treatment [19]. This study found significantly increased MBL levels in Japanese patients approaching end stage renal failure (4343 ng/ml) and on hemodialysis treatment (8879 ng/ml) as compared with normal individuals (1452 ng/ml). All except one patient in our cohort were undergoing renal replacement treatment prior to transplantation. The MBL levels in our patients were similar to those found in normal controls. The reason for this difference is unclear. Since MBL is too large in size to be cleared by glomerular filtration in the non-proteinuric kidney, renal insufficiency by itself would not explain accumulation of MBL.

Genotyping our kidney acceptors for MBL mutations would have allowed classifying patients independently of external factors influencing MBL levels and would have made the use of a somewhat arbitrary MBL cut-off level unnecessary. Unfortunately

DNA was not available for MBL-genotyping of our recipients. On the other hand, although largely genetically determined, MBL levels can vary considerably within one genotype and the actual phenotype probably is functionally more important than the genotype. A comparison with healthy MBL-genotyped donors revealed a close association between MBL variant alleles and MBL serum concentrations below 400 ng/ml. Furthermore recent data from our group indicate that serum MBL levels closely correlate with MBL pathway function [13].

MBL may influence the outcome of a kidney transplant by various mechanisms. The complement system is known to contribute to organ damage in the setting of ischemia/reperfusion [1;2;20]. Inhibition of the lectin complement pathway with an antibody directed against MBL reduces C3 deposition and organ damage in a rat model for myocardial ischemia/reperfusion injury [12]. A recent study has shown MBL to be co-deposited with C6 in both a murine model of ischemia/reperfusion injury and human transplant kidneys after reperfusion [21]. We did not find an association of the MBL levels with delayed graft function as a marker for ischemia/reperfusion damage. This does not exclude that MBL contributes to more subtle forms of ischemia/reperfusion damage than overt acute tubular damage.

Next to ischemia/reperfusion injury the complement system is also involved in the context of acute and chronic rejection. Inhibition of complement activation by administering a membrane-binding complement regulator based on complement receptor type 1 resulted in amelioration of ischemia/reperfusion damage in a rat model of kidney transplantation. This intervention also led to a reduction of acute and chronic rejection [22]. Transplantation of kidneys obtained from C3 knockout mice leads to marked improvement of graft survival when compared with kidneys obtained from wild type mice [23]. In our study higher MBL levels seem to be associated with more severe and possibly treatment-resistant forms of rejection Damage resulting from acute rejection may be enhanced in the presence of high circulating levels of MBL by interaction of MBL with damaged tissue. MBL can bind to necrotic and late apoptotic cells, resulting in enhanced phagocytosis of these cells by macrophages and dendritic cells [11;24;25]. Phagocytosis of necrotic cells may induce dendritic cell maturation and macrophage activation [26-28]. It is conceivable that high MBL levels may increase immune reactivity and cell damage via binding to damaged tissue and enhancing activation of antigen presenting cells. The observation that higher MBL levels are associated with more treatment-resistant forms of rejection necessitating additional courses of rejection treatment after application of antithymocyte globulin may point to a higher prevalence of humoral rejection in high MBL individuals.

Initially the interest in the lectin complement pathway was directed towards beneficial effects in the setting of infectious diseases. The high frequency of polymorphisms in the MBL gene however points towards a possible advantageous effect of low MBL levels [29]. This concept is strengthened by recent data. In a mouse model of acute septic peritonitis MBL-A deficient mice had enhanced survival [30]. MBL mutations associated with low MBL serum levels may protect against the development of ulcerative colitis in humans [31]. In type 1 diabetics high MBL genotypes have been associated with an increased frequency of diabetic nephropathy [32]. In the same population the presence of cardiovascular disease was associated with higher MBL levels. The lectin complement pathway has also been linked to renal damage in IgA nephropathy and Henoch-Schonlein purpura [15,33,34].

In conclusion our study suggests that higher MBL levels are associated with poorer graft survival due to rejection-associated graft loss in deceased donor kidney transplantation. Obviously this observation will need confirmation in other transplantation cohorts. At this point of time we can only speculate about the mechanisms responsible for this deleterious effect of MBL. Further studies into the role of MBL in ischemia/reperfusion injury of the kidney and the interaction of the complement system with cellular immune mechanisms may help to understand this interesting finding. In addition to the potential pathophysiological implications of these findings our data suggest that determining MBL levels prior to transplantation may serve as a prognostic marker in kidney transplantation.

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