Low mannose-binding lectin and increased complement activation correlate to allograft vasculopathy, ischaemia, and rejection after human heart transplantation

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KEYWORDS
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Aims Transplant-associated coronary artery disease (TxCAD) is a major cause of post-transplant graft failure. The aim of this study was to investigate a possible role of mannose-binding lectin (MBL) deficiency and complement activation in TxCAD.

Methods and results In a prospective study of heart transplant recipients (n = 38) with a follow-up of 5.3 ± 1.3 years (range: 0.9–6.6), angiographically verified TxCAD (n = 6) was correlated to plasma MBL, complement activation, and endothelial activation (soluble E-selectin). MBL deficiency (<100 ng/mL) was detected in 3/6 patients with TxCAD and in 3/32 with non-TxCAD (Kaplan–Meier, P = 0.020). Furthermore, one or more acute rejection episodes were observed in 6/6 of the MBL-deficient patients and in 15/32 of the MBL-sufficient patients (χ²; P = 0.016). Complement activation (C4bc) correlated with soluble E-selectin (r = 0.36; P = 0.027), both being significantly higher in patients with ischaemia detected in the first biopsy (C4bc: 13.4 ± 6.1 AU/mL; E-selectin: 96 ± 13 ng/mL) than in those without ischaemia (C4bc: 6.3 ± 0.5; E-selectin: 51 ± 6; P = 0.037 and 0.002). Finally, terminal complement complex correlated closely with mortality (P = 0.002).

Conclusion Low MBL was related to the development of TxCAD and acute rejection and increased complement activation correlated to histopathologic ischaemia and mortality after heart transplantation.

Introduction
Transplant-associated coronary artery disease (TxCAD) is an important factor in the development of graft failure and late survival after heart transplantation.1 The vascular pathology that underlies TxCAD appears to be initiated by an endothelial cell activation and inflammation2 that contributes to post-operative ischaemia-reperfusion injury,3,4 graft rejection, and failure after allotransplantation.5

Data from animal studies indicate that activation of complement, in particular the terminal pathway, plays a direct role in developing the vascular inflammation that leads to TxCAD.2 For example, IgM and complement contributed to accelerated atherosclerosis in an aortic allograft rat model.6 Also, Qian et al.7 showed that normal rats developed more pronounced TxCAD and chronic graft failure after heterotopic heart transplantation than rats deficient in complement factor C6, lesions starting in smaller arteries before affecting larger ones. Although findings suggest that the terminal complement pathway is of importance for the development of experimental TxCAD, the possible role of complement in the pathogenesis of TxCAD in humans is at present unclear.

Deficiency of mannose-binding lectin (MBL), the initiator of the complement lectin pathway and an essential component of the innate immune system, has been associated with severe atherosclerosis8 and with the development of coronary artery disease (CAD) in patients with antibodies to Chlamydia pneumoniae.9 However, it is not known whether MBL deficiency is associated with TxCAD.

The main aim of the present prospective clinical study was therefore to evaluate a possible role for MBL and activation of the complement system in the development of early TxCAD in humans, correlating the activation of complement...
to biopsy-verified ischaemia and acute rejection. We here demonstrate for the first time that low MBL concentration is associated with TxCAD and acute rejection and that increased complement activation correlates with histopathologic ischaemia and mortality.

Methods

Patient characteristics and clinical parameters

A total of 38 patients were included in the period 1997–98. They were followed prospectively for 5.3 ± 1.3 years (range: 0.9–6.6) with blood tests the first year. Conventional triple immunosuppression consisting of prednisolone, ciclosporin A, and azathioprin was used in all patients. The demographic data for the patients and donors are shown in Table 1. There were no differences in patient or donor characteristics between those with and those without TxCAD.

Myocardial biopsies

Myocardial biopsies were performed weekly the first 8 weeks after transplantation, thereafter at week 12, 26, and annually for 3 years. Histopathology was graded according to the International Society for Heart and Lung Transplantation Guidelines (0: no rejection; 1–3: weak to severe rejection).

Angiography

Coronary angiography was performed annually by a series of injections of contrast agents in carefully chosen angulated views. The diagnosis of TxCAD was based on the presence of coronary luminal irregularities in one of the main arteries. The angiograms were analysed by one of the authors blinded for the results of the blood tests.

Serum and plasma samples

Blood, collected at 3, 5, 8, 12, 26, and 52 weeks after transplantation, was drawn into pyrogen-free tubes without any additives (serum) or with ethylenediaminetetraacetic acid (EDTA) as anticoagulant (plasma). The tubes were immediately placed in crushed ice, centrifuged (1000 g, 10 min) within 15 min (plasma) or after coagulation (serum). Plasma and serum were stored at -80°C until analysed. Serum was used for all analyses except for the complement activation products. The latter were measured in EDTA-plasma. The samples were thawed less than three times. All the samples obtained were analysed for all the variables described subsequently. All patients were followed for 6 months, whereas one patient died between 6 and 12 months.

MBL antigen and function

The concentration of MBL was quantified by a double antibody enzyme-linked immunosorbent assay as described previously. Lower detection limit was 15 ng/mL. The function of MBL was measured according to a previously described method. This assay detects the function of MBL as well as the MBL-associated serine proteases in the serum sample. Pre-transplant serum samples were not included in the original protocol, but stored serum samples taken prior to transplantation were available in 14 patients. These sera had randomly been collected and importantly, the clinical and haemodynamic status in these patients before transplantation was not different from those whose serum samples were not available. Of these 14 sera, 4 were MBL deficient, including the sera of 3 who developed TxCAD. The MBL concentration in the pre-transplant samples was identical to the post-transplant values, indicating that the MBL concentration was not influenced by the transplantation. MBL deficiency was defined as a concentration of MBL <100 ng/mL.

Classical and alternative complement products

C1q (classical pathway) and factor B (alternative pathway) were quantified using assays for radial immunodiffusion performed according to the manufacturer’s instruction (The Binding Site Ltd, Birmingham, UK). C3 and C4 were quantified by nephelometry (Dade Behring, Marburg, Germany).

Complement activation products

The following assays were performed principally as previously described: C1rs–C1-inhibitor complexes (C1rs-C1inh) from the classical pathway, C4bc reflecting classical as well as MBL pathway, the alternative pathway C3 convertase C3bBbP, C3bc indicating activation of any initial pathway, and the soluble terminal complement complex (TCC) indicating complete activation of the terminal pathway. The results for all assays are given in arbitrary units (AU) per millilitre on the basis of fully activated serum (heat-aggregated IgG for C1rs-C1inh and C4bc, and zymosan for the remaining) defined to contain 1000 AU/mL. The antibodies to C1-inhibitor and C4bc were a kind gift from Prof. C.E. Hack, Amsterdam, The Netherlands.

C-reactive protein

High-sensitivity C-reactive protein (hsCRP) was measured by Tinaquant (Roche Diagnostica, Basel, Switzerland) particle-enhanced immunoturbidimetric assay performed on Roche Hitachi 917 (Roche).

Endothelial cell activation

Soluble E-selectin, a specific marker for endothelial cell activation, was quantified using an enzyme-linked immunosorbent assay performed according to the manufacturer’s instruction (Bender MedSystems, Vienna, Austria).

Statistics

For paired data, univariate repeated-measures ANOVA was performed a priori with time and grouping variable as fixed factors, including subject number as a random factor to compensate for within subject effects. Data that were not normally distributed at baseline were logarithmically transformed prior to inclusion in the general linear model. Paired or unpaired non-parametric tests for two samples were used when appropriate (if ANOVA significant).

Table 1 Patient and donor characteristics (n = 38)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>51 ± 9</td>
</tr>
<tr>
<td>Men [%]</td>
<td>35 (92)</td>
</tr>
<tr>
<td>Aetiology: CAD [%]</td>
<td>20 (53)</td>
</tr>
<tr>
<td>Diabetes mellitus pre-HTx [%]</td>
<td>4 (10)</td>
</tr>
<tr>
<td>Hypertension pre-HTx [%]</td>
<td>5 (13)</td>
</tr>
<tr>
<td>Hypercholesterolemia pre-HTx [%]</td>
<td>8 (21)</td>
</tr>
<tr>
<td>Follow-up time (years; median and range)</td>
<td>5.3 (0.9–6.6)</td>
</tr>
<tr>
<td>Total cholesterol (52 weeks)</td>
<td>5.9 ± 1.1</td>
</tr>
<tr>
<td>Post-HTx (mmol/L)</td>
<td>80 ± 80</td>
</tr>
<tr>
<td>Acute rejection (all grades) [%]</td>
<td>21 (55)</td>
</tr>
<tr>
<td>Donor characteristics</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>32 ± 11</td>
</tr>
<tr>
<td>Men [%]</td>
<td>22 (58)</td>
</tr>
<tr>
<td>Ischaemic time of organ (min)</td>
<td>123 ± 67</td>
</tr>
<tr>
<td>Cause of death (CVA, BHI, anoxia) (n)</td>
<td>31/4/3</td>
</tr>
</tbody>
</table>

Data are given as mean ± SD unless otherwise specified. BHI, blunt head injury; CVA, cerebrovascular accident; HTx, heart transplantation.

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Proportions were analysed with the \( \chi^2 \) test. Correlations were calculated using Spearman’s rank correlation coefficient. Survival data are presented as Kaplan–Meier curve with corresponding hazard ratio (HR). The data in Table 1 were analysed by Mann–Whitney (continuous variables) or \( \chi^2 \) (binary variables) test. Data are given as mean ± standard error of the mean. The level of statistical significance was chosen as \( P < 0.05 \). All tests are two-sided.

Ethics

The study complies with the Declaration of Helsinki, the locally appointed Ethics Committee approved the research protocol, and informed consent was obtained from the subjects.

Results

Development of TxCAD

**MBL and TxCAD**

Of the 6 patients developing TxCAD, 3 were MBL deficient as defined by values <100 ng/mL (36–66 ng/mL), whereas only 3 of 32 non-TxCAD patients were MBL deficient (51–71 ng/mL) \( (P = 0.020, \text{HR} = 5.40, \text{Kaplan–Meier}) \) (Figure 1). In contrast to MBL, no deficiencies of the classical and alternative complement proteins C1q, factor B, C3, and C4 were detected in the patient population. Thus, the correlation between development of TxCAD and the results obtained from analysis of the 3 week samples were restricted to MBL. MBL concentration and MBL function correlated significantly \( (r = 0.34; P = 0.039) \).

**Rejection and ischaemia**

**MBL and acute rejection**

A total of 36 acute rejection episodes were observed in 21 patients. Acute rejection was seen more frequently in patients with MBL deficiency. All 6 of the MBL-deficient patients developed one or more acute rejection episodes compared with 15 of the 32 without MBL deficiency. The difference was statistically significant \( (\chi^2; P = 0.016) \). The acute rejections were observed early in the observation period (35/36 during the first 12 weeks).

**Complement activation, ischaemia in the first biopsy, and endothelial activation**

Complement activation is related to ischaemia and endothelial cell activation. Consistently we found that complement activation, as detected by \( C4bc \), was significantly higher in the group with histopathologic ischaemia in the first biopsy \( (13.4 \pm 6.1 \text{ AU/mL}) \) than in those without ischaemia \( (6.3 \pm 0.53 \text{ AU/mL}) \) \( (P = 0.037) \) (Figure 2). Furthermore, the soluble E-selectin concentration was significantly \( (P = 0.002) \) higher in the ischaemia group \( (96 \pm 13 \text{ ng/mL}) \) than in those without ischaemia \( (51 \pm 6 \text{ ng/mL}) \) (Figure 2). There was a significant correlation between the values of \( C4bc \) and E-selectin \( (r = 0.36; P = 0.027) \). In contrast, there was no difference in the classical pathway, C1rs–C1-inhibitor complexes, between the ischaemic and non-ischaemic groups.

**12 months follow-up data on acute-phase reaction and mortality**

**MBL and acute-phase reaction**

MBL has been regarded as an acute-phase protein. However, there was no correlation between MBL and hsCRP in the present study. Although the MBL concentration did not change during the observation period (Figure 3A), the hsCRP value increased 3 weeks after transplantation \( (10.2 \pm 2.3 \text{ mg/L}) \) and thereafter gradually decreased to \( 3.0 \pm 1.0 \text{ mg/L} \) at 52 weeks (Figure 3B).
Complement activation and mortality
Mean TCC concentration during the follow-up correlated closely ($P = 0.002$; Kaplan–Meier) to mortality as 4 of 5 patients who died had TCC $0.39$ AU/mL (third inter-quartile value), whereas this was the case for only 1 of 33 of the survivors ($\text{HR} = 22.4 \ (2.1–244)$) (Figure 4) indicating that persistently increased complement activation during the course was associated with fatal outcome.

Discussion
MBL is a central recognition pattern protein of the innate immune system with a relatively high frequency of genetic deficiency.13–15 MBL deficiency with low serum concentration is associated with increased susceptibility to infection by extracellular microbes,16 whereas low MBL levels possibly protect against infections with certain intracellular pathogens.17,18 Furthermore, MBL deficiency seems to be a disadvantage in some autoimmune diseases,19,20 and herein we show that MBL deficiency also may be harmful by predisposing to development of TxCAD. These findings may seem in contrast with other reports showing that MBL deficiency reduced the inflammatory response after severe ischaemia-reperfusion in humans10 and that anti-MBL-neutralizing antibodies protected against experimental acute myocardial ischaemic injury. Thus, MBL seems to play a dual role in human pathophysiology. On one hand it is important for human defence and tissue homeostasis, and on the other it is harmful when activating complement inappropriately. The exact role of MBL deficiency in various disorders will have to be further elucidated.

In the present study both MBL serum concentration and function were measured. There is a close relation between the concentration of MBL and genetic variants.18 The genotypes 0/0 and XA/0 are regarded as MBL deficient and correspond to levels $<100 \text{ ng/mL}$, which is usually defined as cut-off for MBL deficiency.22 The findings of a higher proportion of MBL deficiency and abolished MBL function in TxCAD patients, together with the close correlation between the serum concentration and function of MBL, further strengthen the conclusion that MBL deficiency predisposes to TxCAD and acute rejection. Results obtained from pre-transplant serum samples from 14 of the 38 patients, including 4 with MBL deficiency, revealed that the MBL concentration was not influenced by the transplantation.

MBL has not been previously studied in patients with TxCAD. However, our data are in agreement with a recent study demonstrating an association between the development of native CAD and MBL deficiency.9 Interestingly, the latter study showed that MBL deficiency per se was not sufficient for the development of CAD, as the correlation was dependent on simultaneous presence of antibodies to C. pneumoniae. Thus, a possible effect of MBL deficiency leading to CAD could be increased susceptibility to infection. This may also be a possibility in the development of TxCAD, as it has recently been shown that the presence of antibodies against C. pneumoniae is associated with development of TxCAD.23

CRP, which is the prototype of an acute-phase protein, is a candidate for classical pathway activation in myocardial ischemic damage24 and there is increasing evidence that CRP-mediated complement activation participates in the pathogenesis of atherosclerosis.25 Many of the complement
proteins are acute-phase reactants as well and MBL has, from animal studies, been suggested to behave as an acute-phase protein. However, in the present study we found that in contrast to the typical CRP response after transplantation with a gradual decrease to normal values, there was no change in MBL levels throughout the study period.

Complement plays an essential role in ischaemia-reperfusion damage26,27 and there is a close interplay between the activation of complement and endothelial cell activation.28 Increased soluble E-selectin, a specific marker of endothelial activation, has previously been shown in patients developing congestive heart failure,29 whereas increased complement activation is part of the pathophysiology of heart failure.20 In the present study we found a correlation between C4bc and soluble E-selectin in the whole patient material. The fact that C4bc was increased but C1rs–C1-inhibitor complexes remained normal may indicate a lectin pathway-mediated activation. Independent of the mechanism, the data suggest that complement activation goes with activation of the endothelium, the key event in any pathologic process in the heart after transplantation. C4bc and soluble E-selectin were significantly related to the histopathologic demonstration of ischaemia in the first biopsy after transplantation. Interestingly, the same correlation was not found for ischaemic time of the organ (data not shown), indicating that ischaemia detected histopathologically reflects actual ischaemia, whereas the ischaemic time does not. This is not surprising because ischaemic changes may have occurred prior to donor organ withdrawal, and organ storage conditions other than time obviously influence the status of the organ at the time of transplantation. It has gradually become evident that protection of early endothelial cell activation is essential for the long-term survival of an allograft. The present data add to the growing body of evidence that complement activation is harmful for the endothelium in general and for the allograft endothelium in particular. The fact that mortality was closely correlated to the degree of complement activation should be interpreted with caution because of the low number of patients, but is nevertheless of interest as systemic complement activation in general reflects major disturbances in pathophysiology.

Two limitations should be considered when interpreting the present data. First, the study population is relatively small and a limited number of patients developed TxCAD. This limitation is partly counteracted by the prospective design of the study, consecutive inclusion of all patients transplanted during the study period and the long follow-up period. Secondly, the diagnosis of coronary disease was based on angiography. Intravascular ultrasound, which is suggested to be more sensitive than angiography for the diagnosis of TxCAD, was not established at the time when the study was conducted. Thus, larger prospective studies in the further ultrasound diagnosis of CAD will be needed to confirm the present findings and to further elucidate the role of MBL and complement in the development of human TxCAD.

In conclusion, the present data are the first to correlate MBL deficiency to development of TxCAD. Furthermore, acute rejection was related to MBL deficiency, whereas histopathologic ischaemia and mortality were correlated with increased complement activation.

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