TITLE

Circulating anti-human leukocyte antigen IgM antibodies as a potential early predictor of allograft rejection and negative clinical outcome after lung transplantation

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ABSTRACT

Purposes: Anti-human leukocyte antigen (HLA) immunoglobulin (Ig) M production stimulated by an alloantigen is sensitive, thus making IgM a potential and novel marker of allorejection after organ transplantation. This study aimed to examine the relationship between serum levels of anti-HLA IgM early after clinical lung transplantation (LTx) and post-transplant outcomes.

Methods: Thirty-one consecutive patients who underwent deceased LTx were included. Immunoreactivity against HLA was retrospectively analyzed by measuring anti-HLA IgM of the serum sampled for the first 14 days after LTx. The flow panel reactive antibody technique was used. The ratio of the anti-class I IgM level at each day to baseline was obtained, and a peak IgM was determined for each case. Correlation between the peak IgM level and subsequent development of acute rejection (AR), chronic lung allograft dysfunction (CLAD), and survival outcomes were examined.

Results: The peak IgM level was a significant risk factor for AR within 90 days in univariate and multivariate analyses. In the long term, the patients with positive IgM (peak level > 1.8) tended to have a poorer CLAD-free and overall survival.

Conclusion: Elevation of anti-HLA IgM levels early after LTx potentially correlated with a higher incidence of rejection and worse clinical outcomes.

MAIN BODY

Introduction

Despite the recent establishment of a basic methodology for immunosuppressant management, chronic lung allograft dysfunction (CLAD) remains a major limitation to long-term survival for lung transplant (LTx) recipients. According to the international database, bronchiolitis obliterans syndrome (BOS), a typical phenotype of CLAD, is the leading morbidity and cause of mortality for >1-year survivors after LTx [1]. It is recognized that multifactorial events in the early post-transplant period, which are associated with both natural and acquired immunity, are involved in the subsequent chronic rejection process [2-5]. Of these, the alloimmune response provoked by mismatched HLA antigens plays a pivotal role in the development of chronic allograft dysfunction. However, there is no early prognostic predictor or a monitoring method to optimize the personalized adjustment of immunosuppression [6].

A growing number of recent studies have focused on donor-specific alloantibody (DSA) as a cause of reluctant antibody-mediated rejection (AMR) and irreversible allograft dysfunction. Most studies have identified the actual impact of DSA and subclinical or clinical AMR on CLAD or patients' survival [7-10]. Post-transplant de novo DSA discussed here was categorized as class immunoglobulin G (IgG) and generally regarded as one of the clinical markers for poor prognosis. However, the timing of problematic elevation of serum de novo DSA-IgG levels reportedly varies widely and is mostly at more than one month after LTx. Once

DSA-IgG emerges, furthermore, the elimination and treatment of rejection require painstaking processes and often fail. These facts suggest that DSA-IgG does not necessarily work effectively as an early clinical marker to detect reluctant acute rejection or CLAD in the preventable or treatable stage.

We previously reported that donor-specific immunoglobulin M (IgM) levels in the recipient's serum were promptly elevated post-transplant in response to acute rejection in the clinical setting of bilateral living-donor LTx [11]. Production of IgM from plasma cells is triggered by a primitive antigen exposure process. The responses of DSA-IgM were specifically observed in a very early stage, prior to the development of clinical signs of acute rejection (AR) in the study. IgM production is sensitive to immunoreaction and is stimulated theoretically in accordance with the extent of reactivity against donor antigens [11-13]. This hypothesis supports the hypothesis that elevated levels of recipient serum IgM directed to human leukocyte antigen (HLA) can be a sensitive marker of immunoreaction against allograft and early predictor of long-term graft survival after LTx. This study aimed to examine the relationship between serum levels of anti-HLA IgM early after LTx and subsequent post-transplant outcomes.

Materials and Methods

Patients and study design

A total of 31 patients who underwent deceased-donor LTx between July 2013 and July 2016 at Okayama University Hospital were included in the study. Plasma samples from these patients were obtained and preserved once pretransplant and on days2, 4, 7, 10, and 14 after transplantation. In addition, the medical records of the patients were reviewed to ascertain the independent variables determining the clinical characteristics and post-transplant outcomes. Serum samples and clinical data were obtained following approval of the institutional review board of Okayama University Hospital (approval #: 1609-026) and consent from the patients.

Statistical analyses were conducted to explore the relationship between patient variables, including acute IgM levels and subsequent acute rejection events within 90 days after LTx. Furthermore, the study cohort was divided into IgM-positive group and IgM-negative groups based on the cutoff level determined by a receiver operating characteristic curve analysis. The incidence of CLAD and the long-term survival were then evaluated and compared between the groups.

Recipient and Donor selection

Patients who had officially approved indications for LTx were registered on the waitlist provided by the Japan Organ Transplant Network (JOTN). Procedure indication was determined

for each candidate according to the primary disease, urgency, and organ availability. The patients in the cohort had no previous sensitized history. Offered deceased-donor lungs were allocated to recipients according to waitlist order, ABO compatibility, matching of predicted pulmonary function value, and negative result of lymphocyte crossmatching test (complement-dependent cytotoxicity test for warm T and B cells). Detailed donor data, including past medical history and examination results, were obtained by authorized donor coordinators and shared among the donor hospital staff and transplant centers with the use of a form filled out by the authorized donor coordinators and transplant physicians [14]. Our institutional expert team made a final decision to accept or decline the donor organs.

Transplant procedure and post-transplant management

The lung procurement procedure was standardized for all deceased donors. The donors' lungs were routinely flushed with EP-TU extracellular solution (Cell Science & Technology Institute, Sendai, Japan). LTx surgery was performed in a standard manner via a transsternal anterior thoracotomy approach. Intraoperative cardiopulmonary support with a standard bypass technique was used when the unilateral native lung was not able to maintain adequate gas exchange and hemodynamic stability. Recipients received a standard triple-drug immunosuppressive regimen post-transplant consisting of a calcineurin inhibitor (tacrolimus), a

cell-cycle inhibitor (mycophenolate mofetil, MMF), and steroids (prednisolone). The target trough level of tacrolimus was set at 8-11 ng/mL. The doses of MMF and prednisolone were optimized according to the patient's body weight. The prednisolone tapering policy was standardized.

Detection of Anti-HLA IgM antibodies after LTx

Recipient serum IgM with an affinity for the HLA panel was retrospectively analyzed at the Central Research Laboratory, Okayama University Medical School. Blood samples were obtained on days 2, 4, 7, 10, and 14 after LTx, and preserved following centrifugal separation. Flow cytometry crossmatching was performed with the use of flow panel reactive antibody (PRA) screening test HLA class I beads (One Lambda) referring to the manufacture's instruction for IgG detection. Briefly, 5 μL of the beads were mixed with 20 μL of sera and incubated for 30 min. Then, secondary antibody to IgM (Jackson ImmunoResearch), instead of IgG, was added and reacted to the composites for 30 min to label the anti-class I HLA IgM caught by the panel beads. Fluorescence intensity was analyzed using a MACS Quant Analyzer (Milteny Biotec). The mean fluorescence intensity (MFI) for overall panel beads reacted to the IgM was calculated. All the process required two hours per single examination.

Definition for data interpretation

The MFI value of day 2 sample for the flow PRA IgM testing was defined as the baseline anti-HLA IgM level. Then, the ratio of the MFI values on the following post-transplant days to baseline was calculated. Through the paired sera examination, the maximal value of the ratio of the first 14 days after LTx was adopted as representative data for the acute anti-HLA IgM level for each case. AR was diagnosed cautiously by experienced transplant clinicians based on the following clinical findings: (1) elevation of an inflammatory marker (C-reactive protein); (2) deteriorated oxygenation; (3) ground-glass opacities, peribronchovascular infiltration and interlobular septal thickening detected by high-resolution computed tomography; (4) increased serous pleural effusion; and (5) the clinical manifestations above were well responsive to pulse steroid treatments. To determine the pre-transplant severity of each patient, the US lung allocation score (LAS) was retrospectively calculated (in November 2016) using the LAS calculator available on the OPTN website (https://optn.transplant.hrsa.gov/resources/allocation-calculators/las-calculator/). Primary graft dysfunction (PGD) grade and the diagnosis of CLAD (BOS and restrictive phenotype) were defined in accordance with the International Society for Heart and Lung Transplantation working group statement [15,16].

Statistical Analysis

Cox regression analysis was used to examine the influence of various patient factors on the development of AR within 90 days after LTx and CLAD. The rate of freedom from CLAD and overall survival were depicted using Kaplan–Meier analysis and compared between the IgM-positive and negative groups using the log-rank test. A p-value <0.05 was considered statistically significant. All analyses were performed using SPSS 24.0.

Results

Patient characteristics and anti-HLA antibody levels early after transplantation

Overall, 155 samples from 31 recipients were analyzed to examine anti-HLA IgM levels in the acute phase. The patient characteristics are shown on Table 1. The cohort comprised 15 women and 16 men. The mean age was 38.4 (range, 8–61) years. The indications for transplantation were interstitial lung disease (n = 10, 32.2%), bronchiolitis obliterans (n = 7, 22.6%), pulmonary hypertension (n = 3, 9.7%), chronic obstructive pulmonary disease (n = 1, 3.2%), and others (n = 6, 19.4%). Ten patients (32.2%) had sensitization history such as pregnancy and blood transfusion. The mean HLA mismatch number was 4.3. Pretransplant MFI levels of anti-HLA IgM ranged from 0.75 to 14.74 (mean, 2.62). In order to contrast the patient's IgM reactivity, we defined the MFI rise rate as the IgM level in each case (MFI value

174 divided by individual baselines). Eventually, peak IgM levels (maximal rise rate) ranged from
175 0.81 to 2.52 (mean, 1.47) in this patient cohort. Transitional changes in the average IgM levels
176 are shown in Figure 1A. There was a general upward trend in the IgM rise rate peaking around
177 10 days after LTx.

178 An example course of anti-HLA IgM transition in a representative case is depicted in
179 the Case1, Figure 2. The patient was a 33-year-old man with obstructive pulmonary disease. He
180 underwent right single LTx and subsequently developed steroid-responsive acute rejection on
181 postoperative day 8. The peak IgM level in this case was regarded as 2.46.

182

183 **Impact of peak anti-HLA IgM level on the development of AR and CLAD**

184 Seven patients developed AR during the first 90 days after LTx. All the IgM data for
185 the patients with AR are demonstrated in Figure 2. The onset of AR was found from sixth to
186 sixtieth posttransplant day (mean 17.43 days after LTx). The influence of relevant major clinical
187 factors on AR development within 90 days after LTx was analyzed (Table 2). The peak IgM
188 level was a significant risk factor with a hazard ratio of 15.642 in univariate analysis ($p=0.001$).
189 In multivariate analyses, the peak value of the IgM rise rate was an independent factor
190 predicting subsequent development of AR. As for the analysis of the impact on CLAD (Table 3),
191 the peak IgM early after LTx exhibited a much higher hazard ratio (3.3) than other clinical

variables, although this difference was not statistically significant (p=0.069). No other items had evident influence.

Analysis for cutoff value determination and comparison between positive and negative IgM groups

A receiver operating characteristics curve analysis determined 1.8 as the cutoff anti-HLA IgM level for AR development (Figure 3). Patients were then divided into two groups according to their values. Seven patients had positive anti-HLA IgM levels (1.8 times or higher elevation) and 24 were negative (< 1.8).

Transitions of the average IgM levels in the positive and negative groups are shown in Figure 1B. The positive IgM group exhibited a substantially wider fluctuation in the IgM level than the negative IgM group. A comparison between the positive and negative anti-HLA IgM groups is shown in Table 4. Six of 7 patients in the positive group had AR episodes, while only 1 of 24 developed AR in the negative group (p=0.001). There was a higher proportion of interstitial pneumonia as the primary disease for LTx in the positive group (p=0.04). There was no association of PGD grade or other postoperative inflammatory events with anti-HLA IgM elevation.

Long term outcomes by anti-HLA IgM levels

The median observation period of the survivors was 63 months (range, 42–88 months). Patients in the positive anti-HLA IgM group developed CLAD more frequently (p=0.03) (Figure 4A). Four of 7 patients in the positive group developed CLAD, while 4 of 24 patients in the negative group developed CLAD. As for mortality after LTx, the positive IgM group had poorer survival outcomes than the negative group, although the difference did not reach statistical significance (p=0.17) (Figure 4B). Three of the 7 recipients with positive IgM died during the observation period; the cause of death in all the patients was CLAD, one of which developed comorbid reluctant acute AMR episodes.

Discussion

Although the immunologically risky pairing of donor and recipient (e.g., evidence of preformed DSA or positive lymphocyte crossmatching test) is usually precluded in the common organ allocation policy, the challenge of management against frequent acute and chronic rejection is still inevitable after LTx. Susceptibility of the transplanted organs to rejection varies by HLA differences between the donor and recipient. However, it is common for every post-transplant management to start and continue immunosuppressant treatment with a standardized dose and concentration according to a generalized protocol. The dosage of

228 immunosuppressants is usually modified and customized after the development of the clinical
229 signs of rejection or drug side effects. This is mainly attributed to the fact that there is no early
230 clinical marker in use to predict how likely rejection develops later in each transplant case. This
231 study focused on anti-HLA IgM production in the recipient early after LTx to address this issue.
232 We explored serum IgM not from an aspect of actual damaging factor but as a prognostic
233 marker in LTx recipients in this study.

234 We identified an increase in the risk of AR, CLAD, and mortality in patients with
235 anti-HLA-IgM surge early after LTx. Patients with more than 1.8-fold (approximately double)
236 increase in anti-HLA IgM during the first two weeks after LTx had higher risks of subsequent
237 graft rejection than those with less than double. The IgM rise was determined by means of
238 paired-serum examination (proportion to baseline level) because patients could originally have
239 natural anti-HLA or cross-reactive IgM, which influenced the baseline values on an individual
240 basis. The starting immunosuppressant management policy was standardized during the study
241 period with equal target trough levels of tacrolimus and steroid tapering policy. De novo
242 HLA-IgM production is largely unaffected by various other inflammatory events such as
243 primary graft dysfunction. Overall, the rise in anti-HLA IgM can be one of the early predictors
244 of subsequent immune reactivity and graft survival.

245 The detailed mechanism of the association between humoral immune response and T

246 cell mediated rejection or chronic rejection is not well known. However, there are considerable
247 clinical evidence previously reported, indicating potentially close correlation among them.
248 According to a study of a large-scale series of kidney transplantations, acute rejection episodes
249 were significantly associated with the development of de novo DSA [17]. Furthermore, other
250 recent studies demonstrated that the combination of T cell and antibody mediated rejection is
251 much frequently observed in the condition of chronic allograft failure rather than purely
252 mechanized rejection [18-20]. Cellular immunity induced by CD4+ Th2 cells can prompt
253 specific B cells to produce DSA during acute rejection [21]. The Th1 and Th2 cells mutually
254 inhibit each other's activity through cytokine signals but can coexist. We previously reported the
255 early elevation of circulating immunoglobulin (Ig) M levels against donor lymphocytes in
256 histologically proven acute cellular rejection in rat allo-LTx models [12, 13]. Those studies
257 suggested that a dynamic humoral immune response occurs even in cellular rejection and links
258 to chronic allograft failure. In addition, it is well recognized that repeated acute rejection early
259 after LTx is associated with higher risk of later development of bronchiolitis obliterans
260 syndrome [22]. The early fluctuation of anti-HLA IgM we observed was a primitive but
261 sensitive change that could represent general immune reactivity against the allograft antigenicity.
262 We consider that monitoring early immune response after transplantation would be a reasonable
263 way to predict and grade the subsequent immunological risk after transplantation.

IgM is the first isotype of immunoglobulin that emerges in the blood after initiation of antigen recognition. It is controversial whether donor specific IgM has functionality to injure the graft by itself and the specificity of IgM is equivalent to IgG. However, its serum levels sensitively rise in response to donor antigenicity prior to class switching. IgM against donor HLA was similarly detected in kidney and heart transplant recipients with undermined survival outcomes [23]. As for the characteristics of the major histocompatibility complex (MHC) antigen of the graft, class I is widely expressed on the graft tissues and recognized as an alloantigen very early after transplantation [24,25]. Although de novo antibodies against MHC class II antigens mainly contribute to persistent rejection and graft dysfunction in the long-term [26], upregulation of class II antigen provoking an immunoreactivity can occur after some processes of graft injury. Theoretically, therefore, antibodies toward MHC class II usually emerge later than antibodies toward class I [25]. We did not find significant fluctuations in IgM toward class II antigens during the first 14 days after LTx in a preliminary study (data not shown). Overall, it is reasonable to consider that elevation of IgM levels against class I HLA can be an early sensitive maker of subsequent rejection and graft survival.

We employed the flow PRA technique to screen anti-HLA IgM in this study. Although a flow PRA screening kit was originally used for IgG detection and qualitative analysis, the methodology to transfer it to an anti-HLA IgM assay was reported previously [27]. In the

framework of the flow PRA test used in this study, it was not proven whether the increased anti-HLA IgM components were truly donor-specific or not. However, the specific antibody to the specific donor HLA alleles is not targetable to quantify as the most effective marker because the types of HLA antigens that provoke early responsive antibody production are unpredictable. In addition, repeated examination to search for the peak levels for specific antibodies (with a single antigen technique) is quite expensive. Therefore, it is a logical and practical way to monitor the whole anti-HLA IgM value by applying the flow PRA method as shown in this study.

There are several limitations inherent to the design of our study. It is retrospective and small-scale in nature. Dose management for immunosuppressants after rejection development was not standardized and conducted in the clinical judgment on an individual case basis. Donor specificity of the increased IgM was not validated to simplify the monitoring methodology as mentioned above. The negativity of pretransplant anti-HLA IgG was determined by the conventional complement-dependent cytotoxicity method. The study was focused on the impact of anti-HLA IgM as a very early sensitive maker of rejection rather than its specificity and the effect of de novo IgG was not evaluated here. The qualitative methodology of conventional flow PRA technique was transferred to the way to measure MFI of the overall anti-HLA IgM titer. Furthermore, the diagnosis of AR was made clinically but not histologically. It is necessary to

take them into consideration when interpreting the study results.

In conclusion, elevation of anti-HLA IgM levels early after LTx potentially correlated with a higher incidence of later rejection and worse outcomes. Monitoring the level of anti-HLA IgM early after transplant can contribute to subsequent optimal immunosuppressive adjustments. However, whether the IgM value can reflect the immunoreactivity similarly in the chronic phase remains an open question. A further study to observe IgM-positive recipients and determine how to manage immunosuppressant management is warranted.

AUTHOR CONTRIBUTIONS

Kazuaki Miyahara, MD: Data collection and analysis, statistics, drafting article

Kentaroh Miyoshi, MD, PhD: Clinical practice, concept / design, data interpretation, drafting and revising the article

Takeshi Kurosaki MD, PhD: Clinical practice, data collection

Shinji Otani MD, PhD: Clinical practice, data collection

Seiichiro Sugimoto MD, PhD: Clinical practice, data collection

Masaomi Yamane MD, PhD: Clinical practice, data collection, critical revision of the article

Shinichi Toyooka MD, PhD: Clinical practice, data interpretation, critical revision of the article

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Conflict of interest statement:

Kazuaki Miyahara and other co-authors have no conflict of interest.

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403 **Legends**

404 Figure 1. Transition of average anti-HLA IgM level in the overall cohort (A), negative and
405 positive-IgM groups (B). The IgM levels are calculated as rise rates of MFI compared to the
406 baseline of the day 2 MFI level in each case.

407 Figure 2. A monitoring of anti-HLA IgM in seven patients with acute rejection episodes. Steroid
408 pulse therapy was initiated on the day of AR diagnosis. Six of the seven patients (except case 2)
409 exhibited a clear surge of anti-HLA IgM level surpassing 1.8 of rise rate in the first 14-day
410 monitoring period. The IgM levels were determined by means of paired MFI examination where
411 baseline was defined as MFI value of day 2.

412 Figure 3. Receiver operating characteristic curve indicating a cutoff level of anti-HLA IgM for
413 prediction of subsequent acute rejection. Optimal sensitivity and specificity were achieved when
414 the cutoff value was set at 1.8.

415 Figure 4. A Kaplan-Meier analysis for the chronic lung allograft dysfunction free survival (A)
416 and overall survival (B). The positive and negative IgM groups are compared.