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ORIGINAL ARTICLE

Dietary phosphorus restriction by a standard low-protein diet decreased serum fibroblast growth factor 23 levels in patients with early and advanced stage chronic kidney disease

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Abstract
Background Elevated serum fibroblast growth factor 23 (FGF23) levels are associated with mortality, cardiovascular disease, and disease progression in patients with chronic kidney disease (CKD). Although recent studies demonstrated that FGF23 levels decreased in response to dietary restriction of phosphorus and/or use of phosphate binders, research on the effects of a standard low-protein diet is lacking.
Methods The effects of a standard low-protein diet on serum FGF23, intact parathyroid hormone, and 1,25-dihydroxyvitamin D levels were investigated in patients with early ($n = 15$) and advanced ($n = 20$) CKD.
Results Serum FGF23 levels decreased in both groups. Changes in FGF23 levels correlated with changes in 24 h urinary phosphorus excretion in the advanced CKD group. Decreased serum intact parathyroid hormone levels were observed only in the advanced CKD group and increased serum 1,25-dihydroxyvitamin D levels only in the early CKD group.
Conclusions These findings suggest that consuming standard low-protein diet decreased serum FGF23 levels in patients with CKD. Serum FGF23 levels may therefore be a useful marker to monitor the effects of a low-protein diet in early and advanced stage CKD.

Keywords Chronic kidney disease · Fibroblast growth factor 23 · Protein restriction

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Introduction

Fibroblast growth factor 23 (FGF23) is a phosphaturic hormone that plays an important role in phosphorus metabolism. Recent clinical studies reported that high serum FGF23 levels are associated with mortality, cardiovascular events, and disease progression in patients with chronic kidney disease (CKD) [1–9]. A previous experimental study demonstrated that increased serum FGF23 levels can directly induce cardiac hypertrophy [10]. Thus, high serum FGF23 levels are seen as a risk factor in patients with CKD.

FGF23 is produced in bone and regulated by several factors, especially phosphorus intake. Several studies reported that serum FGF23 levels increase in response to dietary phosphorus overload, and dietary phosphorus restriction decreases serum FGF23 levels in healthy subjects [11–13]. Many studies reported that dietary phosphorus restriction and/or use of phosphate binders can decrease serum FGF23 levels [14–20] in patients with CKD; however, others failed to demonstrate this effect [21, 22]. Although a standard low-protein diet is widely used in treatment of patients with CKD, the effect of such a diet on circulating FGF23 levels has not yet been investigated.

FGF23 is also regulated by 1,25-dihydroxyvitamin D (1,25D) [23, 24]. In patients with advanced CKD, 1,25D synthesis decreases; thus, the effect of phosphorus intake on circulating FGF23 levels may differ between early and advanced disease stages. Sigrist et al. [14] demonstrated that serum FGF23 levels decrease in response to dietary phosphorus restriction in both healthy controls and patients with CKD. However, most patients in that study had stage 3 CKD, and only 2 had stage 4 disease. Therefore, the difference in the effects of phosphorus intake on circulating FGF23 levels between early and advanced CKD remains

unclear. Hence, a comparison of the effects of phosphorus intake in early and advanced stages of CKD may provide valuable insight into the control of CKD.

We aimed to investigate the effects of dietary phosphorus restriction by a standard low-protein diet on FGF23 levels in patients with early and advanced CKD. We also assessed the effects of the diet on other FGF23-related factors.

Methods

Study population and design

We recruited consecutive patients with early (stages 1 and 2) and advanced (stages 4 and 5) CKD who were hospitalized between March 2010 and August 2012 in the Nephrology and Kidney Center, Kobe University Graduate School of Medicine (Kobe, Japan). Written informed consent was obtained. Early CKD was defined as an estimated glomerular filtration rate (eGFR) $\geq$60 mL/min/1.73 m^2 and proteinuria or hematuria for at least 3 months before study initiation. Advanced CKD was defined as eGFR <30 mL/min/1.73 m^2 for at least 3 months before study initiation. eGFR was calculated using the equation for Japanese patients recommended by the Japanese Society of Nephrology [25]. Exclusion criteria were as follows: use of active vitamin D sterols, history of dialysis or kidney transplantation, or rapid deterioration of kidney function. Patients were also excluded if they were unable to consume the full study meals or could not provide written informed consent. This study was conducted in accordance with the principles of the Declaration of Helsinki, and the study protocol was approved by our institutional review board (Approval No.: 1009). This study is registered with the UMIN Clinical Trials Registry (Registration No.: UMIN000003049).

All participants consumed a regular diet for 2 days, followed by a low-protein diet for 4–6 days. The regular diet provided 1.1–1.4 g/kg/day of protein, 15–20 mg/kg/day of phosphorus, 600–800 mg/day of calcium, and 30–35 kcal/kg/day. The low-protein diet provided 0.6–0.8 g/kg/day of protein, 10–15 mg/kg/day of phosphorus, 600–800 mg/day of calcium, and 30–35 kcal/kg/day. Fasting blood samples and 24 h urine samples were obtained on the final day of the regular diet and low-protein diet periods.

Measurements

Patient data regarding age, gender, height, and weight were collected at enrollment. Serum calcium, phosphorus, albumin, and creatinine levels as well as urine phosphorus levels were measured by standard laboratory techniques using automatic analyzers. Serum intact parathyroid hormone (PTH) levels were measured using an electro chemiluminescence immunoassay (Elecsys PTH; Roche Diagnostics, Mannheim, Germany). Serum FGF23 levels were measured using the intact human FGF23 enzyme-linked immunosorbent assay (Kainos Laboratories, Inc., Tokyo, Japan). Serum 1,25D levels were measured using a TFB 1,25-dihydroxyvitamin D radioimmunoassay kit (Immunodiagnostic Systems Ltd., Boldon, UK). Serum calcium levels were corrected for albumin concentration using the original Payne's equation [26].

Statistical analysis

Continuous variables are presented as mean $\pm$ standard deviation or median (interquartile range). Categorical variables are presented as percentages. Demographic and laboratory data were compared between the early and advanced CKD groups using the unpaired Student's t test and Chi-squared test, where appropriate. Changes in laboratory data were compared using the paired Student's t test. In cases of skewed data, analyses were performed after log-transformation. Pearson's correlation test was used to examine relationships between changes in 24 h urinary phosphorus excretion and changes in serum FGF23, intact PTH, and 1,25D levels. $P < 0.05$ was considered statistically significant. All analyses were performed using Dr. SPSS II for Windows, version 11.01 J (SPSS Japan, Tokyo, Japan).

Results

Patient characteristics

41 patients were initially enrolled in this study. However, 4 withdrew their consent, 1 started undergoing dialysis, and 1 violated the study protocol; therefore, 35 patients were included in the final analysis (early CKD group, $n = 15$; advanced CKD group, $n = 20$). The characteristics of patients included in this study are listed in Table 1. In the advanced CKD group, patients were significantly older than those in the early CKD group were and included a higher percentage of males. No patient had diabetes in the early CKD group, whereas 45 % patients had diabetes in the advanced CKD group. Serum calcium levels were comparable among groups. Serum phosphorus, PTH, and FGF23 levels were significantly higher and serum 1,25D and urinary phosphorus levels were significantly lower in the advanced CKD group than in the early CKD group. Eleven patients in the early CKD group and 14 in the advanced CKD group consumed a low-protein diet for 5 days, 4 patients in the early CKD group and 5 in the advanced CKD group consumed a low-protein diet for 4 days, and 1 patient in the advanced CKD group consumed a low-protein diet for 6 days.

Table 1 Clinical and biochemical characteristics of patients

	Early CKD ($n = 15$)	Advanced CKD ($n = 20$)	P
Age (years)	45 ± 13	66 ± 13	<0.001
Male, *n* (%)	2 (13)	13 (65)	0.005
eGFR (mL/min/1.73 m^2)	79.1 ± 14.2	14.1 ± 8.4	<0.001
cCa (mg/dL)	9.3 ± 0.5	9.1 ± 0.4	0.08
P (mg/dL)	3.8 ± 0.3	4.5 ± 0.1	0.008
Intact PTH (pg/mL)	23.0 (19.0–27.0)	123.5 (72.8–193.5)	<0.001
1,25-dihydroxyvitamin D (pg/mL)	36.0 (33.0–39.0)	14.5 (13.0–19.0)	<0.001
FGF23 (pg/mL)	52.8 (36.0–74.9)	185.4 (125.5–321.6)	<0.001
Urinary P (mg/day)	604 ± 170	456 ± 189	0.02

CKD Chronic kidney disease, *cCa* corrected calcium, *eGFR* estimated glomerular filtration rate, *FGF23* fibroblast growth factor 23, *P* phosphorus, *PTH* parathyroid hormone

Effects of a standard low-protein diet on biochemical parameters

Changes in serum FGF23, PTH, and 1,25D levels are shown in Fig. 1. Serum FGF23 levels significantly decreased in both groups [early CKD, from 52.8 (36.0–74.9) to 38.6 (23.0–65.1), $P = 0.006$; advanced CKD, from 185.4 (125.5–321.6) to 138.2 (96.7–257.8), $P = 0.005$; Fig. 1a]. The duration of a low-protein diet did not affect changes in serum FGF23 levels (4 days vs. 5 days; early CKD, -13.5 ± 22.4 vs. -12.4 ± 15.6, respectively, $P = 0.913$; advanced CKD, -27.8 ± 62.0 vs. -38.6 ± 61.3, respectively, $P = 0.742$). Serum PTH levels significantly decreased in the advanced CKD group, but remained unchanged in the early CKD group [early CKD, from 23.0 (19.0–27.0) to 25.0 (21.0–30.0), $P = 0.89$; advanced CKD, from 123.5 (72.8–193.5) to 85.0 (61.3–168.3), $P = 0.001$; Fig. 1b]. Serum 1,25D levels significantly increased in the early CKD group, but remained unchanged in the advanced CKD group [early CKD, from 36.0 (33.0–39.0) to 47.0 (33.0–78.0), $P = 0.03$; advanced CKD, from 14.5 (13.0–19.0) to 15 (9–17), $P = 0.12$; Fig. 1c].

Table 2 shows changes in serum-corrected calcium and phosphorus levels, urinary excretion of phosphorus, and eGFR. Serum-corrected calcium levels remained unchanged in both groups. Serum phosphorus levels significantly decreased in the advanced CKD group, but remained unchanged in the early CKD group. Urinary phosphorus levels significantly decreased in both groups. No change in eGFR was observed in either group.

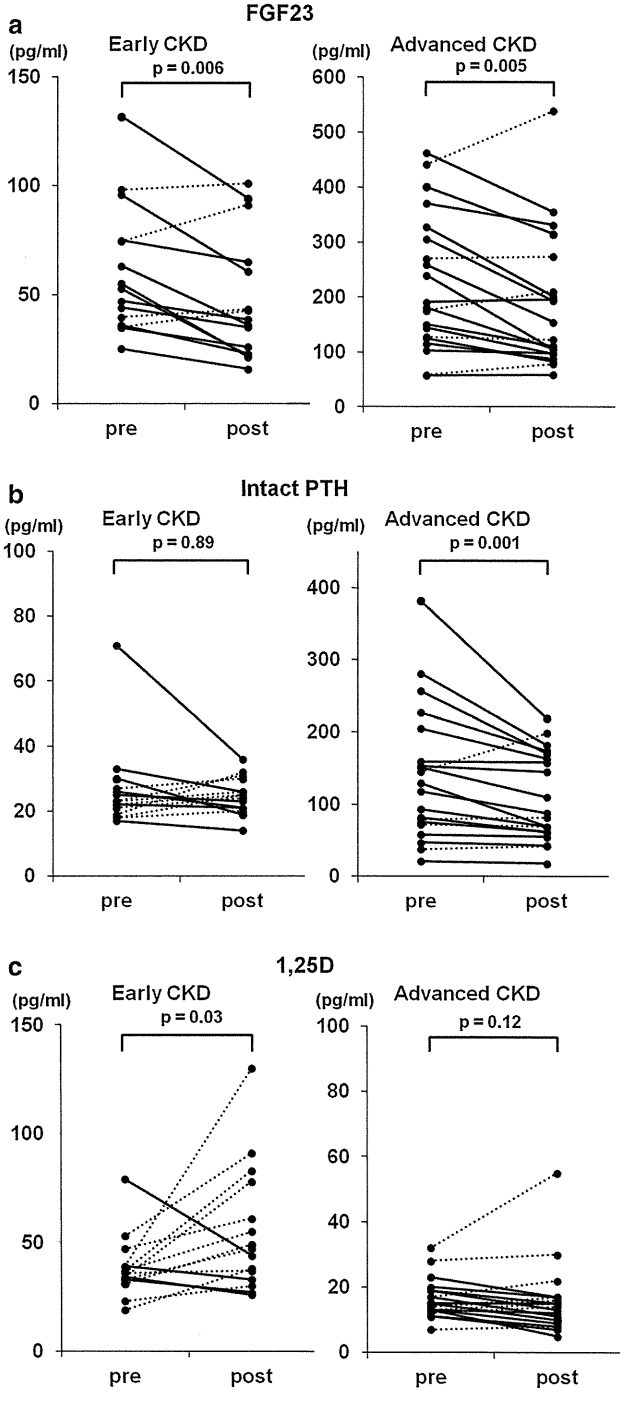

Fig. 1 Effects of a standard low-protein diet on serum. **a** FGF23, **b** intact PTH, and **c** 1,25D levels. *Solid lines* indicate cases in which values for these parameters decreased, and *dotted lines* indicate cases in which values for these parameters increased

Correlations among changes in serum FGF23, PTH, 1,25D, and urinary phosphorus levels

Figure 2 shows correlations among changes in serum FGF23, PTH, and 1,25D as well as urinary phosphorus. Significant correlations were found between changes in

Table 2 Effects of a standard low-protein diet on serum-corrected calcium and phosphorus levels, urinary phosphorus levels, and estimated glomerular filtration rate

	Early CKD ($n = 15$)			Advanced CKD ($n = 20$)		
	Pre	Post	*P*	Pre	Post	*P*
cCa (mg/dL)	9.3 ± 0.5	9.3 ± 0.5	1.00	9.0 ± 0.5	9.1 ± 0.4	0.17
P (mg/dL)	3.8 ± 0.3	3.7 ± 0.4	0.26	4.5 ± 1.0	4.2 ± 0.9	0.01
Urinary P (mg/day)	604 ± 170	437 ± 176	0.02	456 ± 189	411 ± 170	0.02
eGFR (mL/min/1.73 m^2)	79.1 ± 14.2	81.4 ± 18.6	0.19	14.1 ± 8.4	14.5 ± 8.8	0.22

CKD Chronic kidney disease, *cCa* corrected calcium, *eGFR* estimated glomerular filtration rate, *P* phosphorus

serum FGF23 levels and changes in urinary phosphorus excretion in the advanced CKD group; however, no such correlation was found in the early CKD group (Fig. 2a). In addition, there were no correlations between changes in serum PTH and 1,25D levels and changes in urinary phosphorus excretion in either group (Fig. 2b, c). Finally, there were no correlations between changes in serum FGF23 levels and changes in serum PTH and 1,25D levels in either group.

Discussion

In this study, decreased serum FGF23 levels were detected in patients with early and advanced CKD who consumed a standard low-protein diet. In addition, serum intact PTH levels decreased only in patients with advanced CKD and serum 1,25D levels increased only in patients with early CKD after consuming a standard low-protein diet.

Recently, 2 studies reported the effects of dietary phosphorus restriction on serum FGF23 levels in predialysis patients: Sigrist et al. [14] in a crossover trial, found that serum FGF23 levels were lower in patients on low-phosphorus diets (750 mg/day) than in those on high-phosphorus diets (2000 mg/day). Another crossover study by Di Iorio et al. [18] revealed decreased serum FGF23 levels in patients with stage 3 and 4 CKD on a very low-protein diet (0.3 g/kg/day) compared with those on a low-protein diet (0.6 g/kg/day). These findings are consistent with our results. However, dietary interventions in these previous studies considerably deviated from actual clinical practice in patients with CKD. The actual phosphorus intake (2529 ± 291 mg/day) in patients on a high-phosphorus diet in the study by Sigrist et al. [14] was considerably higher than the usual phosphorus intake in a normal population consuming a typical Western diet (1500 mg/day) [27]. The very low-protein diet in the study by Di Iorio et al. [18] is also not generally used for treatment of CKD because of concerns regarding malnutrition. In contrast, protein and phosphorus restriction levels in our study were in accordance with the standard recommended intake (protein, 0.6–0.8 g/kg/day; phosphorus, 10–15 mg/kg/day). Therefore, the results of this study suggest that even a change from a regular diet to a standard low-protein diet, which is close to that used in clinical practice, can decrease serum FGF23 levels in patients with CKD. Furthermore, these findings indicate that serum FGF23 levels may be a useful marker of low protein in the diet. Although a low-protein diet is widely used as part of the treatment regimen for CKD, rates of adherence to this diet are often low. At present, 24 h urine collection is necessary to verify the ingestion of a low-protein diet. Measurement of serum FGF23 levels may therefore present a good alternative marker of adherence to low-protein diet.

Although many experimental and clinical studies have shown that phosphorus intake affects serum FGF23 levels, the mechanism by which phosphorus intake regulates FGF23 production has not yet been fully elucidated. An in vitro study showed that phosphorus did not stimulate FGF23 production in osteoblast cell cultures [28]. Although changes in serum FGF23 levels have been reported as a result of long-term dietary phosphorus intervention, no changes in serum FGF23 levels were observed after either dietary phosphorus intervention for several hours [29, 30] or phosphorus injection [31]. In addition, previous clinical studies have demonstrated that changes in serum FGF23 levels due to dietary phosphorus intervention may occur with no changes in serum phosphorus levels [11–13, 15, 16]. In our study, decreased serum FGF23 levels were recorded in the early CKD group despite the fact that serum phosphorus levels were unchanged. These results suggest that phosphorus intake may indirectly affect FGF23 production through other mechanisms.

Excess FGF23 in the body may be a risk factor of disease progression in patients with CKD [1–9]. Gutierrez et al. [1] demonstrated an independent association of mortality with high serum FGF23 levels at initiation of hemodialysis. A similar association was shown between high serum FGF23 levels and mortality in predialysis patients and in patients on maintenance hemodialysis [2–4]. Other observational studies have shown an association

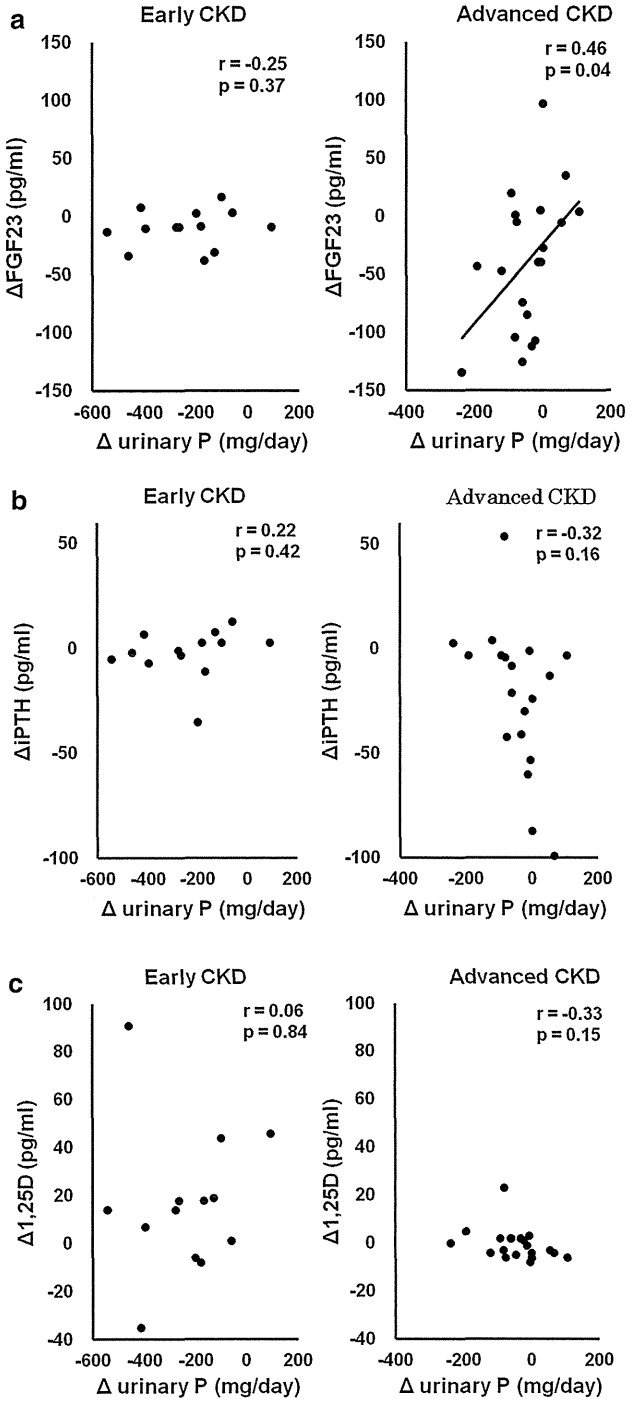

Fig. 2 Correlation between 24-h urinary phosphorus excretion and serum. **a** FGF23, **b** intact PTH, and **c** 1,25D levels

between elevated FGF23 levels, left ventricular hypertrophy, [10, 32] and cardiovascular events [4–6] in patients with CKD. In an experimental study, Faul et al. [10] reported that FGF23 itself may have a direct cardiac hypertrophic effect. In that study, intramyocardial or intravenous injection of FGF23 resulted in left ventricular hypertrophy in wild-type mice, and treatment with an FGF receptor blocker attenuated left ventricular hypertrophy using 5/6 nephrectomy rats. Another recent interventional study showed that treatment with sevelamer, a phosphate-binding agent used to treat hyperphosphatemia, reduced serum FGF23 levels and induced improvement in flow-mediated vasodilatation in patients with stage 4 CKD [17]. Taken together, these findings suggest that elevated serum FGF23 is associated with progression of cardiovascular disease and that decreasing serum FGF23 levels may prevent cardiovascular disease in patients with CKD. The results of this study demonstrated that a standard low-protein diet decreases serum FGF23 levels in patients with early and advanced stages of CKD. Thus, dietary phosphorus may contribute to the association between FGF23 and clinical outcomes in CKD patients.

Relatively few studies have compared the effects of phosphorus restriction on serum FGF23 levels in early stage CKD to advanced stage CKD. A previous study demonstrated that the magnitude of the absolute change in serum FGF23 levels caused by phosphorus restriction was larger in the CKD group than in the control group [14]. In our study, the absolute change in serum FGF23 levels also tended to be larger in the advanced CKD group than in the early CKD group. The effect of dietary phosphorus restriction on the absolute change in serum FGF23 levels may differ between patients with early and advanced CKD. The effect of a standard low-protein diet on serum intact PTH and 1,25D levels also differed between patients with early and advanced CKD. A standard low-protein diet decreased serum intact PTH levels in patients with advanced CKD, but not in those with early CKD. Because serum intact PTH levels of most patients in the early CKD group were within normal limits in this study, we concluded that these levels might not have decreased in response to a standard low-protein diet. Serum 1,25D levels increased only in patients with early CKD. Because the ability to activate 1-α hydroxylase may be remarkably impaired in patients with advanced CKD, no effect of a standard low-protein diet on serum 1,25D levels may occur.

Some studies have demonstrated that FGF23 interacts with PTH in animal models [33, 34]. However, there was no correlation between changes in FGF23 and intact PTH in either group in the present study (early CKD, $r = -0.01$, $P = 0.97$; advanced CKD, $r = -0.21$, $P = 0.30$). To the best of our knowledge, an association between the changes FGF23 and intact PTH has not been previously reported in a clinical study that investigated changes in serum FGF23 levels by dietary intervention or use of phosphate binders. Because the number of participants in our study was relatively small, significant correlations may not be demonstrated. Therefore, larger clinical

trials are necessary to investigate the association between changes in FGF23 and intact PTH serum levels.

In this study, phosphorus excretion was lower in patients with advanced CKD (Table 1). A study by Shigematsu et al. [35] reported that urinary phosphorus excretion was lower in patients with a creatinine clearance <30 mL/min than in patients with a creatinine clearance > 30mL/min. The precise mechanism for the reduction of urinary phosphorus excretion remains unknown. Although phosphorus excretion was maintained by PTH and FGF23 in patients with early CKD, urinary phosphorus excretion may decrease in advanced CKD because phosphorus excretion per nephron reaches maximum levels in advanced CKD.

Here, we demonstrated a correlation between changes in FGF23 and urinary phosphorus excretion in advanced CKD, but not in early CKD. Ferrari et al. [11] demonstrated that changes in FGF23 correlated with changes in urinary phosphorus excretion in 58 samples from a healthy population. Therefore, we hypothesize that there exists a correlation between changes in FGF23 and changes in urinary phosphorus excretion in patients with early CKD. However, because there were only 15 patients in the early CKD group, a larger study may detect a correlation.

Furthermore, we assessed adherence to the study protocol by urinary phosphorus excretion and daily reviews of returned meal containers. However, because intestinal absorption or maximum kidney excretion levels may influence urinary phosphorus excretion, this parameter will vary despite the same phosphorus intake. Other dietary intervention studies also showed that urinary phosphorus excretion varied [11, 13, 14, 18]. To assess oral phosphorus intake more precisely, a further study to assess fecal excretion in a closed facility is needed.

This study had some limitations. First, the study period was relatively short. Because serum FGF23 levels changed 1 day after dietary intervention [12], we believe that the study period was sufficient to investigate the effects of a low-protein diet on serum FGF23 levels. However, in order to assess the long-term effect of a low-protein diet on serum FGF23 levels, a longer interventional trial is needed. Second, our results were not clear as to whether serum FGF23 levels were decreased by only phosphorus restriction or both phosphorus restriction and protein restriction. Phosphorus restriction has been reported to decrease serum FGF23 levels. However, it remains unknown whether protein restriction can decrease serum FGF23 levels. Moe et al. [36] demonstrated that a diet in which protein is mainly derived from plants led to lower serum FGF23 levels than a diet with protein mainly derived from meat. Therefore, protein restriction may also decrease serum FGF23 levels; however, further studies are needed to investigate this suspicion. Finally, clinical profiles differed between patients in the early and advanced CKD groups. Therefore, further studies are necessary to validate that absolute change in serum FGF23 by a low-protein diet and the effect of low-protein diet on serum intact PTH and 1,25D levels differ between patients with early and advanced stage CKD.

In conclusion, our results showed that the administration of a standard low-protein diet decreased serum FGF23 levels in patients with early and advanced CKD, suggesting that serum FGF23 may be a useful marker of a low-protein diet.

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Conflict of interest The authors declare that no conflicts of interest exist.

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Late-onset neutropenia and acute rejection in ABO-incompatible kidney transplant recipients receiving rituximab and mycophenolate mofetil

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ABSTRACT

Introduction: Using rituximab, we have performed successful ABO-incompatible kidney transplantations in recipients without splenectomy as well as in those with high pretransplant anti-A/B antibody titers. A common and increasingly recognized toxicity of rituximab is late-onset neutropenia (LON), defined as unexplained grades III to IV neutropenia occurring at least 4 weeks after the last dose of rituximab in the absence of an alternative explanation.

Patients and methods: Between May 2006 and December 2011, 25 patients who received rituximab underwent successful ABO-incompatible kidney transplantation and were enrolled as the subjects in this study. The incidence rate and clinical features of LON as well as the relationship between LON and acute rejection in these patients were studied.

Results: Twelve recipients (48%) experienced LON 2 to 12 months after transplantation. Five of the 12 patients (41.6%) who developed LON had an episode of biopsy-confirmed acute cellular rejection, as compared with one of the 13 patients (7.7%) who did not develop LON. Moreover, 3 patients who experienced LON developed steroid and deoxyspergualin-resistant acute cellular rejection requiring OKT-3 administration.

Conclusions: The frequency of acute cellular rejection was higher in ABO-incompatible kidney transplant recipients with LON than in those without LON. Our findings suggested that these recipients who developed LON after rituximab administration may be at an increased risk for acute cellular rejection.

1. Introduction and hypothesis

Due to the severe shortage of deceased donors in Japan, ABO-incompatible kidney transplantation has been performed since the late 1980s. Recently, a new avenue in the management of ABO-incompatible kidney transplantation has been demonstrated by the introduction of an anti-CD20 monoclonal antibody, rituximab [1,2]. Rituximab, a chimeric monoclonal antibody against human CD20 antigen, lyses CD20-positive cells in antibody- and complement-dependent cell killing processes [3]. It has been used in desensitization protocols for ABO-incompatible kidney transplantations [1,2] and as rescue therapy for humoral rejection [4,5].

A common and increasingly recognized toxicity of rituximab is late-onset neutropenia (LON), defined as unexplained grades III to IV neutropenia occurring at least 4 weeks after the last dose of rituximab in the absence of an alternative explanation. The severity of neutropenia is classified based on the absolute neutrophil count. Absolute neutrophil counts in the range of 500–1000 cells/μl and lower than 500 cells/μl are classified as grade III and grade IV, respectively [6,7].

A previous report demonstrated that patients who received rituximab had a rate of acute rejection that was higher than the rate previously observed among patients who had not received induction therapy. Depletion of immunoregulatory B cells may have contributed to this increased rejection in the rituximab-treated patients [8]. In addition, a recent study showed a marked elevation of serum B cell activating factor (BAFF) levels in kidney transplant recipients with LON. BAFF, also known as B lymphocyte stimulator, is a molecule of the tumor necrosis factor (TNF) family that functions as a key regulator of peripheral B cell populations and promotes B cell survival [9]. Elevated levels of B cell-related cytokines may therefore play a role in LON and acute rejection after rituximab administration in kidney transplant recipients.

In this present pilot study, we investigated the incidence rate and the clinical features of LON as well as the relationship between LON and acute rejection in ABO-incompatible kidney transplant patients receiving rituximab and mycophenolate mofetil (MMF) administration.

Abbreviations: LON, late-onset neutropenia; BAFF, serum B cell activated factor; TNF, tumor necrosis factor; MMF, mycophenolate mofetil; AMR, antibody-mediated rejection; CMV, cytomegalovirus; G-CSF, granulocyte-colony stimulating factor.

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2. Patients and methods

2.1. Patients

Between June 2006 and December 2011, 82 patients with end-stage renal disease underwent living donor kidney transplantation at Osaka City University Hospital, of which 25 cases were ABO-incompatible transplantation with rituximab induction. All 25 patients who received rituximab underwent successful transplantation with a median current serum creatinine of 1.18 mg/dl (0.61–3.21 mg/dl), and patient and graft survival rates were 100% at the time of this writing. These 25 cases were enrolled as the subjects in this study. The median observation period was 41.2 months (24.9–85.4 months). The median patient age at transplant was 50 years (15–74 years). Graft donation from a spouse was the most frequent (56%), and graft donation from a parent accounted for 36%. The most common ABO blood type of the recipients was B (40%). The most common ABO blood type of the donors was A (48%). The causes of end-stage renal disease were chronic glomerulonephritis (n = 5), renal sclerosis (n = 2), IgA nephropathy (n = 3), diabetic nephropathy (n = 2), other disease (n = 8), and unknown (n = 5). The patients had anti-A/B titers ranging from 1:2 to 1:4096.

LON was defined as grades III to IV neutropenia occurring at least 4 weeks after the last administration of rituximab in the absence of any alternative reason to explain its occurrence [6,7]. The subjects were divided into two groups, the LON (+) group: patients who developed LON and the LON (−) group: patients who did not develop LON.

2.2. Immunosuppression protocols

To remove the anti-A/B antibodies, the patients underwent 4–10 sessions of double filtration plasmapheresis or plasma exchange prior to kidney transplantation until the anti-A/B antibody titers were ≤1:16. For the desensitization protocol without splenectomy, the patients with low anti-A/B titers (<1:512) received two doses of rituximab (150 mg/m^2) at 2 weeks prior to and on the day of transplantation (standard protocol: Fig. 1) [10,11]. The elderly recipients (>65 years) received only a single dose of rituximab (150 mg/m^2) at 2 weeks prior to transplantation to minimize its adverse effects (elderly protocol: Fig. 2) [12]. The patients with high titers (≥1:512) or with donor-specific antibody received both a splenectomy on the day of transplantation and administration of 2 doses of rituximab (high-titer protocol: Fig. 3) [13,14]. The pretransplant immunosuppressive protocol included 4 weeks of MMF 1 g/day for B-lymphocyte suppression. The elderly patients received 0.5 g/day of MMF for 4 weeks. Basic immunosuppression after transplantation consisted of basiliximab, steroid, cyclosporine or tacrolimus, and MMF. Basiliximab was given at a dose of 20 mg on the day of transplantation and on the postoperative day 4. Steroid was administered at a dose of 500 mg on the day of transplantation, and the dose was subsequently tapered. Cyclosporin or tacrolimus was started 3 days before the operation and continued throughout the postoperative course. Cyclosporin was given to maintain a blood trough level of 250–300 mg/dl, and tacrolimus was given to maintain a blood trough level of 13–18 ng/ml. The pretransplant dose of MMF was continued postoperatively, but it was reduced to 0.5 g/day at 2 weeks after transplantation for the patients with low anti-A/B titers (<1:512) to avoid over-immunosuppression. These protocols were approved by the Human Ethics Committee of Osaka City University Hospital. All subjects gave informed consent for participation in the study. All the procedures were in accordance with the Helsinki Declaration of 2000.

2.3. Diagnosis and treatment of acute rejection

Rejection episodes were confirmed by histological diagnosis. For the diagnosis of antibody-mediated rejection (AMR), the deposit of C4d in the peritubular capillaries was identified by immunofluorescence microscopy. Protocol biopsy after renal transplantation was performed within 3 months. Each time rejection was suspected, an episode biopsy was performed. Acute cellular rejection and acute AMR were diagnosed based on the Banff 09 classification. For treatment of acute cellular rejection episodes, methylprednisolone was administered at a dose of 500 mg/day alone for 3 days or in combination with deoxyspergualin (5 mg/kg/day; 5–7 days). When resistance to these drugs was confirmed, muromonab CD3 (OKT-3) was administered. For treatment of AMR, plasmapheresis was performed in conjunction with rituximab administration.

2.4. Lymphocyte subset (CD19$^+$ and CD20$^+$) monitoring

Lymphocyte immunophenotyping by flow cytometry was performed and lymphocyte subsets [CD19$^+$ (anti-human CD19 antibody (B4-RD1, COULTER CLONE, Beckman Coulter)) and CD20$^+$ (anti-human CD20 antibody (B1-RD1, CYTO-STAT, Beckman Coulter))] were monitored in all the patients before rituximab infusion and at 1 month, 3 months, 6 months, and 12 months after transplantation.

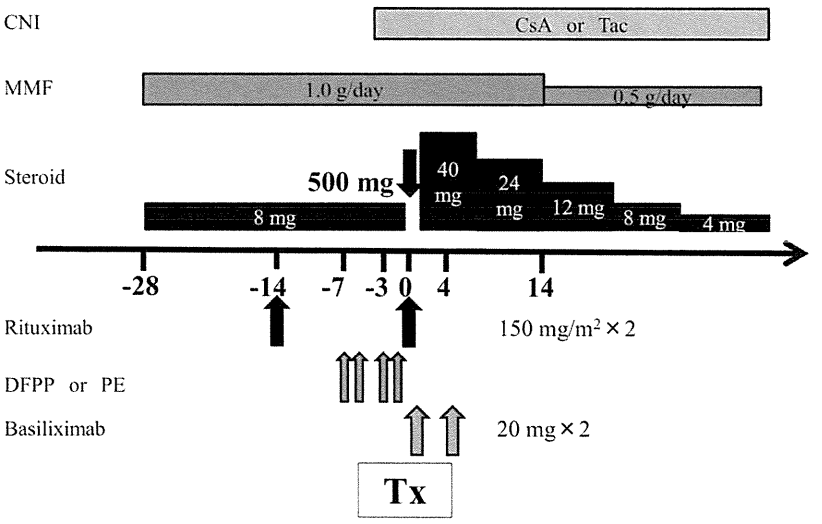

Fig. 1. Immunosuppressive protocol for ABO-incompatible kidney transplantation without splenectomy (standard protocol).

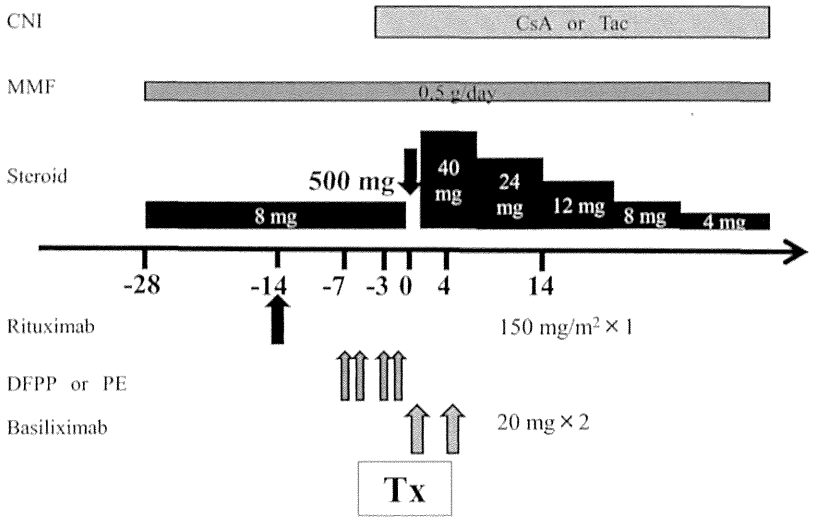

Fig. 2. Immunosuppressive protocol for ABO-incompatible kidney transplantation without splenectomy in elderly recipients (elderly protocol).

2.5. Statistical analysis

All analyses and calculations were performed using the Stat View V Statistical System. The results were presented as median and ranges for continuous variables and as the proportion for the categorical variables. Differences between the groups were examined with Student's *t*-test or Mann–Whitney *U*-test. Categorical variables were compared using chi-squared analysis or Fisher's exact test.

3. Results

3.1. Patient characteristics

Twelve recipients (48%) experienced LON 2 to 12 months after transplantation (LON (+) group), while 13 patients did not develop LON (LON (−) group). Other drugs that could induce myelosuppression excluding MMF and rituximab were not given to the patients in the LON (+) group at neutropenia onset, and the primary causes of neutropenia such as viral and bacterial infections did not occur. The characteristics of the patients in each group are shown in Table 1. There were no significant differences in the age at transplant, gender ratio, cyclosporine/tacrolimus ratio, distribution of the underlying kidney diseases, donor age, or donor relation between the two groups. LON developed in 8 out of the 14 patients receiving the standard protocol as desensitization, in 2 out of the 3 patients receiving the elderly protocol, and 2 out of the 8 patients receiving the high-titer protocol. The number of plasmapheresis sessions did not differ significantly between the two groups.

3.2. Rate of acute allograft rejection episodes

Five of the 12 patients (41.6%) who developed LON had an episode of biopsy-confirmed acute cellular rejection, as compared with one of the 13 patients (7.7%) who did not develop LON, and the frequency of acute cellular rejection was higher in the LON (+) group than in the LON (−) group ($p = 0.0726$). The only patient in the LON (−) group who had acute cellular rejection was classified as Banff IA 59 days after transplantation, which responded to intravenous methylprednisolone and administration of deoxyspergualin. Two of the recipients in the LON (+) group who had acute cellular rejection classified as Banff 1A due to the elevation in serum creatinine levels were successfully treated with steroid pulse therapy and administration of deoxyspergualin. These 2 patients experienced acute rejection 18 and 45 days after transplantation, respectively. However, the 3 other patients who experienced acute cellular rejection in the LON (+) group developed steroid- and deoxyspergualin-resistant acute cellular rejection requiring OKT-3 administration. The onset of intractable acute rejection requiring OKT-3 administration occurred at 18, 210, and 240 days after transplantation, respectively. Although the timing of LON preceded the onset of acute rejection in 2 cases, LON occurred after acute rejection episode in 3 cases. The median days between LON and acute rejection were 78 days (36–

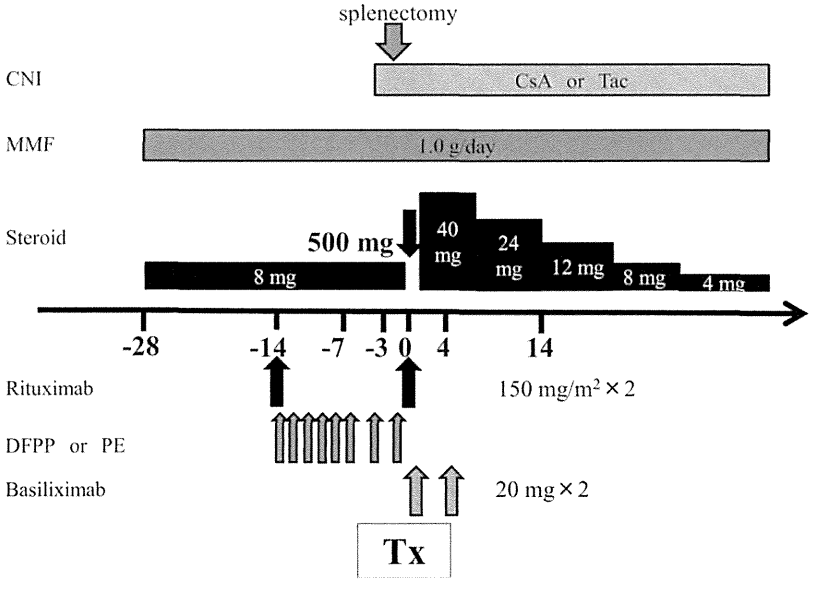

Fig. 3. Immunosuppressive protocol for ABO-incompatible high-titer kidney transplantation (high-titer protocol).

Table 1
Patient characteristics.

	Late-onset neutropenia (−)	Late-onset neutropenia (+)
Patient number	13	12
Age at transplant	52 (23–69)	47 (15–70)
Gender (M/F)	8/5	7/5
Donor	Spouse; 9, parent; 4	Spouse; 5, parent; 5, child; 2
Donor age	55 (45–67)	47 (25–67)
HD duration	50 (4–77)	13 (0–180)
HLA m.m.	4 (1–6)	3.5 (2–5)
ABO-incompatibility	A → O; 5, A → B; 3, B → A; 3, AB → A; 1, AB → B, 1	A → O; 1, A → B; 3, B → O; 2, AB → A; 3, AB → B; 3
CNI (CsA/Tac)	9/4	7/5
Protocol	Standard; 6, high titer; 6 elderly; 1	Standard; 8, high titer; 2 elderly; 2
Origin of ESRD	CGN; 4, IgA nephropathy; 1, renal Sclerosis; 1, nephrosis syndrome; 1, DM nephropathy; 2, unknown; 4	CGN; 1, IgA nephropathy; 2, renal Sclerosis; 1, nephrosis syndrome; 1, Gout kidney; 2, others; 3, unknown; 2
Anti-A/B titer (IgG)	×8–×2048	×2–×4096
Anti-A/B titer (IgM)	×8–×512	×2–×128
Number of plasmapheresis	4 (4–10)	4 (4–10)

HD; hemodialysis, HLA; human leukocyte antigen, m.m.; mismatch, CNI; calcineurin inhibitor, CGN; chronic glomerulonephritis.

97 days) in the LON (+) group (Table 2). The number of AMR episodes after transplantation was seen in only 2 cases: one patient in the LON (+) group experienced AMR due to anti-A/B antibodies and one in the LON (−) group had AMR due to donor-specific antibodies. Both grafts were rescued by plasma exchange and administration of rituximab. Although the prevalence of acute rejection was higher in the LON (+) group than in the LON (−) group, current allograft function was similar between the two groups.

3.3. Adverse events and infections

None of the patients had neurologic symptoms suggestive of progressive multifocal leukoencephalopathy. Five cases each in the LON (+) and (−) groups experienced cytomegalovirus (CMV) reactivation revealed by CMV antigenemia, but no obvious invasive tissue disease occurred. The onset of CMV reactivation differed from that of LON (data not shown).

3.4. Characteristics of LON

Twelve recipients (48%) experienced LON 2 to 12 months after transplantation (Table 2). The median time from transplantation until the onset of neutropenia was 123 days (54–348 days). Four recipients had grade IV LON, and eight had grade III. The median duration of LON (neutrophil count $<1000/mm^3$) was 1 week (range 1–6 weeks). All recipients recovered from LON following granulocyte-colony stimulating factor (G-CSF) administration. However, the number of G-CSF administration sessions varied from one to thirteen. The patients with LON and acute rejection received a median of 4 (1–13) sessions of G-CSF administration and the patients with only LON received a median of 4 (1–9) sessions. LON required temporary discontinuation of MMF in 10 patients. The absolute neutrophil count nadir varied between <0.1 and $908/mm^3$, with a median value of $527/mm^3$ (Table 2).

3.5. Changes in lymphocyte subsets ($CD19^+$ and $CD20^+$)

$CD19^+$ cells and $CD20^+$ cells were both extensively depleted immediately after the first administration of rituximab in the two groups. The mean peripheral levels of CD19+ and CD20+ at the time of the operation were 8.53% and 9.60%, respectively. Only partial recovery of $CD19^+$ and $CD20^+$ cells was noted at 1 year after transplantation. The peripheral blood $CD19^+$ and $CD20^+$ cells at 2 years after transplantation were low in most patients.

4. Discussion

In this study, 48% of the ABO-incompatible kidney transplant recipients who received rituximab and MMF developed LON. Surprisingly, 41.6% of the recipients who developed LON had an episode of biopsy-confirmed acute cellular rejection, as compared with 7.7% of the recipients who did not develop LON, and the frequency of acute cellular rejection tended to be higher in the recipients with LON than in those without LON. Moreover, we observed 3 cases of intractable acute cellular rejection which were treated using OKT-3 in the LON (+) group. Our findings suggested that ABO-incompatible kidney transplant recipients who develop LON after rituximab administration may be at an increased risk for acute cellular rejection.

Clatworthy et al. reported from an open-label randomized control trial conducted in Europe that the rate of acute rejection was higher in the rituximab-treated group than in the daclizumab-treated group, which was followed by the suspension of their study. This report also

Table 2
Characteristics of late-onset neutropenia.

Case	Time to neutropenia after transplantation (days)	Nadir neutrophil count (mm^3)	Treatment	Discontinuation of MMF	Duration of neutrophil count $<1000/mm^3$	Onset of acute rejection after transplantation (days)
1	178	520	G-CSF 5 days	−	1 week	−
2	96	750	G-CSF 1 day	−	1 week	−
3[a]	75	870	G-CSF 13 days	+	6 weeks	18
4[a]	54	908	G-CSF 4 days	+	1 week	18
5	143	0	G-CSF 4 days	+	4 weeks	240
6	81	188	G-CSF 7 days	+	5 weeks	−
7	132	180	G-CSF 1 day	+	1 week	210
8	198	740	G-CSF 4 days	+	1 week	−
9[b]	116	137	G-CSF 9 days	+	1 week	−
10	130	534	G-CSF 4 days	+	1 week	45
11	348	520	G-CSF 1 day	+	2 weeks	−
12[b]	95	650	G-CSF 2 days	+	1 week	−

G-CSF; granulocyte colony stimulating factor, MMF; mycophenolate mofetil.
[a] Patients who received splenectomy.
[b] Elderly patients who received only a single dose of rituximab.

showed that patients who received rituximab had a rate of acute rejection that was higher than the rate previously observed among patients who had not received induction therapy. They also demonstrated that elevated levels of B cell-related cytokines were closely associated with the higher incidence rate of acute rejection in the rituximab-treated group [8]. One possible explanation may be that proinflammatory cytokine release associated with B cell depletion might prime antigen-presenting cells. However, recipients in whom rituximab was administered for desensitization or ABO-incompatible kidney transplantation protocol do not appear to be at an increased risk for acute cellular rejection [9,10]. Any cytokine storm would have been resolved before transplantation by preoperative plasma exchange and corticosteroid therapy, and no increase in the risk of rejection would be expected [8,9].

LON has been reported as an adverse effect of rituximab treatment in recipients of organ transplantation. The mechanism of LON development remains unclear. A recent report demonstrated that kidney transplant recipients with LON showed marked BAFF levels. BAFF, also known as B lymphocyte stimulator, is a molecule of the TNF family that functions as a key regulator of peripheral B cell populations and promotes B cell survival. However, this study showed that BAFF had no relationship with acute rejection [9]. Elevated levels of B cell-related cytokines other than BAFF may play a role in acute rejection and/or LON after rituximab administration in kidney transplant recipients. This may therefore be the reason for the high rate of acute rejection in the recipients with LON in our present study. Moreover, the 3 cases of intractable acute cellular rejection requiring OKT-3 administration may have been caused by such cytokine release. In this study, the patients experienced LON both before and after their acute rejection episodes. The various conditions of a number of B cell-related cytokines may induce LON and/or acute rejection.

A previous report showed that after rituximab administration, peripheral B cells were undetectable in all patients, and that serum levels of cytokines, including tumor necrosis factor-α, interleukin-6, and interleukin-10, were increased after transplantation as baseline values in some of the patients who were treated with rituximab [8]. In this present study, $CD19^+$ cells and $CD20^+$ cells were both extensively depleted immediately after the first administration of rituximab as shown previously. However, serum cytokine levels were not measured in this study. In the future, we must analyze the levels of serum B cell-related cytokines in kidney transplant recipients receiving rituximab between those with and without LON.

The frequency and clinical features of LON due to the administration of rituximab in kidney transplantation had remained unanswered. The incidence of LON in lymphoma patients receiving rituximab is generally in the range of 3–27% [7]. In this present study, 48% of the recipients using rituximab and MMF experienced LON. Previously, LON was observed in 22% of ABO-incompatible kidney transplantation recipients receiving a desensitization protocol which included a single low dose rituximab (200 mg/body) administration [15]. Our present study showed that the number of plasmapheresis sessions did not differ significantly between the LON (+) and LON (−) groups, and there was no relationship between LON development and number of plasmapheresis sessions. In this study, two doses of rituximab combined with MMF 1 g/day, initiated 4 weeks before transplantation except for elderly recipients, were used for desensitization of ABO-incompatible kidney transplantation, which may be a potential risk factor for LON. In addition, 2 of the 3 elderly recipients experienced LON, which suggested that even the protocol consisting of a single dose of rituximab and MMF 0.5 g/day initiated 4 weeks before transplantation may be an increased risk for LON in elderly recipients. Our desensitization protocol may therefore induce a high incidence of LON. Rituximab increases the effects of anti-tumor agents, and there is also the risk of increased side effects from a conventional dosage of MMF which has hematoxicity [16,17]. The synergizing effect of rituximab and MMF may accelerate the depletion of B cells and/or bone marrow toxicity. Efforts must be made to create an effective desensitization therapy that does not induce LON for ABO-incompatible kidney transplant recipients. Everolimus may be a safe and effective alternative for recipients of ABO-incompatible kidney transplantation [18].

In our present study, the patients experienced LON at a median of 123 days (54–348 days) after transplantation. Treatment with G-CSF and/or temporary MMF discontinuation led to the resolution of LON in our patients. One possible explanation may be that treatment of LON with G-CSF might play a role in acute rejection. However, all patients with LON received G-CSF, and there was no significant difference in the number of G-CSF administration sessions between acute rejection (+) and (−) patients [acute rejection (+): 4 (1–13) sessions, acute rejection (−): 4 (1–9) sessions]. Moreover, there were 3 patients who had acute rejection prior to LON in this study. Therefore, our present results could not show a relationship between the treatment of LON with G-CSF and onset of acute rejection.

The present study might have limitations because of the small sample size and the fact that it is a retrospective study. Moreover, B cell-related cytokines that may be closely associated with the higher incidence rate of acute rejection and LON were not evaluated in this study. However, although this is a pilot study, this is the first demonstration of the relationship between LON after rituximab administration and acute rejection in ABO-incompatible kidney transplantation.

In conclusion, 48% of ABO-incompatible kidney transplant recipients who received rituximab and MMF developed LON in this study, and 41.6% of the recipients who developed LON had an episode of biopsy-confirmed acute cellular rejection, as compared with 7.7% of the recipients who did not develop LON. Our findings suggested that recipients who developed LON after rituximab administration may be at an increased risk for acute cellular rejection. To confirm these findings, further prospective cohort trials with a larger number of patients are needed in future.

Note: We would like to note that among the authors, K. Kabei, J. Uchida, T. Iwai, and T. Nakatani designed the study, and J. Uchida and K. Kabei wrote the paper. J. Uchida, N. Kuwabara, and T. Yamasaki participated in the patients' follow-up and collected data, while T. Naganuma, T. Iwai, and N. Kumada analyzed the data.

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Clinical Transplantation

Conversion of stable ABO-incompatible kidney transplant recipients from mycophenolate mofetil with standard exposure calcineurin inhibitors (CNIs) to everolimus with very low exposure CNIs–a short-term pilot study

Uchida J, Machida Y, Iwai T, Kuwabara N, Kabei K, Naganuma T, Kumada N, Kawashima H, Nakatani T. Conversion of stable ABO-incompatible kidney transplant recipients from mycophenolate mofetil with standard exposure calcineurin inhibitors (CNIs) to everolimus with very low exposure CNIs–a short-term pilot study.

Abstract: Background: A recent report has demonstrated that as with mycophenolate mofetil (MMF), everolimus is capable of inhibiting human B-lymphocyte function and activation including B-lymphocyte proliferation, apoptosis, and immunoglobulin production *in vitro*. Everolimus may therefore be used as an immunosuppressant in ABO-incompatible kidney transplantation.
Methods: A three-month pilot study was performed to examine the efficacy and safety of conversion of stable ABO-incompatible kidney transplant recipients from MMF with standard exposure calcineurin inhibitors (CNIs) to everolimus with very low exposure CNIs. Sixteen recipients were enrolled in the study. The patients without acute rejection by graft biopsy were switched from MMF to everolimus with CNI minimization. At three months after conversion, graft biopsies were performed to check for acute rejection and C4d deposition.
Results: Conversion to everolimus with CNI minimization for three months did not induce acute rejection and C4d deposition in all of the ABO-incompatible kidney transplant recipients. A slight elevation of anti-A/B antibody titer occurred in our present study. Everolimus was associated with hyperlipidemia and edema.
Conclusions: These results demonstrated that short-term conversion from MMF to everolimus after one yr post-transplant may be a safe and effective alternate for ABO-incompatible kidney transplant recipients requiring temporary discontinuation of MMF.

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Key words: ABO-incompatible – calcineurin inhibitor minimization – everolimus – kidney transplantation – mycophenolate mofetil

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Conflict of interest: None.

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Kidney transplantation is the treatment of choice for patients with end-stage renal failure, and their survival after transplantation is gradually improving (1). Due to the severe shortage of deceased donors in Japan, ABO-incompatible living donor kidney transplantation has been performed since the late 1980s. Excellent outcomes have been achieved, and the rates of graft survival in these patients are currently similar to those in recipients of ABO-compatible grafts (2, 3). The development of powerful immunosuppressant drug combinations including rituximab has resulted in excellent graft and patient survival, and efforts have been made to overcome the ABO barrier in ABO-incompatible kidney transplantation to avoid acute antibody-mediated rejection (4–8). Mycophenolate mofetil (MMF) is a compound that inhibits antibody production by B cells, and previous reports have shown that the administration of MMF (1 g/d) for four wk before transplantation reduce the episodes of post-transplant humoral rejection in ABO-incompatible kidney

transplantation (3, 9). In fact, MMF may be effective in avoiding the explosive production of anti-A/B antibody. However, the high intensity of desensitization protocols for ABO-incompatible kidney transplantation, consisting of rituximab, MMF, and plasmapheresis, may elevate the risk of infectious complications such as cytomegalovirus (CMV) reactivation (3). Furthermore, discontinuation of MMF due to adverse effects such as intractable CMV infection is highly possible.

Everolimus is a mammalian target of rapamycin inhibitor, providing safe and effective immunosuppression after kidney transplantation. A recent report has demonstrated that as with MMF, everolimus is capable of inhibiting human B-lymphocyte function and activation including B-lymphocyte proliferation, apoptosis, and immunoglobulin production *in vitro* (10). However, there have been no reports on the application of everolimus in ABO-incompatible kidney transplantation. To begin with, we carried out a small-scale pilot study with a three-month observation period to assess the safety of conversion of stable ABO-incompatible kidney transplant recipients from MMF to everolimus in the short term.

Patients and methods

Patients

An open-label design was used to examine the safety of conversion of stable ABO-incompatible kidney transplant recipients from MMF with standard exposure calcineurin inhibitors (CNIs) to everolimus with very low exposure CNIs. The following were the inclusion criteria for conversion: (i) at least one yr after transplantation, (ii) normal or slightly impaired renal function defined as a serum creatinine (S-Cr) value less than 2.0 mg/dL, (iii) receiving rituximab as desensitization protocol, (iv) no acute rejection episodes for more than six months, (v) stable renal function in the last six months, and (vi) normal or slightly increased albuminuria defined as urinary albumin excretion (the ratio of spot urine albumin to Cr) <100 mg/g Cr. We had started using rituximab as a component of the immunosuppressive protocol for ABO-incompatible kidney transplantation since June 2006. A total of 25 patients with end-stage renal disease underwent ABO-incompatible living donor kidney transplantation at Osaka City University Hospital between June 2006 and December 2011. In this prospective study, a total of 16 recipients of ABO-incompatible kidney transplantation at our institution were enrolled. All patients were required to be receiving CNIs with MMF and steroids. CNIs, cyclosporine (CsA), or tacrolimus (Tac) were administered at a dose level that resulted in a blood trough level of 100–120 ng/mL (CsA) or 4–6 ng/mL (Tac). The dosage of MMF and methylpredonizolone was 1 g/d and 4 mg/d, respectively, in all patients.

Methods

At study entry, all patients received a graft biopsy to exclude acute cellular and antibody-mediated rejections. All histologic findings were categorized according to the Banff 09 classification. We then analyzed the C4d staining results for all biopsy specimens. C4d staining was performed by immunofluorescence on frozen sections.

The patients without acute cellular rejection and antibody-mediated rejection diagnosed by graft biopsy were switched from MMF 1 g/d to everolimus 1.5 mg/d in the patients who received CsA (CsA group) (11) or everolimus 3.0 mg/d in the patients who received Tac (Tac group) (12) with dose adjustments from one wk onward to target an everolimus trough level of 3–8 ng/mL. Everolimus trough levels were assessed at one wk and one, two, and three months after conversion. The CNI dose was reduced to 40–60% below baseline values with dose adjustments from one wk onward to a target trough level of 25–50 ng/mL (CsA) or 2–4 ng/mL (Tac). Baseline doses of methylpredonizolone were continued unaltered in all patients. All adverse events were collected. At three months after conversion, graft biopsies were performed to check for acute rejection and/or antibody-mediated rejection. The morphologic features and C4d peritubular capillary staining were evaluated, and the occurrence of acute rejection and/or antibody-mediated rejection episodes was analyzed. At baseline and at one, two, and three months after conversion, peripheral blood lymphocyte subsets ($CD19^+$ and $CD20^+$), plasma immunoglobulin (IgG, IgA, IgM) levels, and anti-A/B antibody titers were monitored. The anti-A/B IgM titer was measured using the saline agglutination technique, and anti-A/B IgG titer was measured using the indirect Coombs' test.

At baseline, clinical parameters including age, gender, body weight, body height, cause of end-stage renal disease, duration of dialysis, time to relative transplantation, donor age, donor relation, ABO-incompatibility, number of HLA mismatch antigens, and pre-operative anti-blood type antibody titers were collected. At baseline and at one, two, and three months after conversion, fasting blood samples were obtained in the early morning for biochemical studies, including S-Cr, total

cholesterol, triglycerides, high density lipoprotein cholesterol, low density lipoprotein cholesterol, and trough levels of CNI. Estimated glomerular filtration rate (eGFR) was calculated using the modified Modification of Diet in Renal Disease equation using the new Japanese coefficient (13). Urinary albumin excretion (the ratio of spot urine concentrations of albumin to creatinine) was measured at baseline and at one, two, and three months after conversion. The effects of everolimus with CNI minimization on renal function, urinary albumin excretion, and lipid profiles were examined. All subjects provided written informed consent prior to enrollment in this study, which was approved by the Human Ethics Committee of Osaka City University Hospital. All procedures were in accordance with the Helsinki Declaration of 1975.

Statistical analysis

All analyses and calculations were performed using the Stat View V Statistical System. The results were presented as mean values ± standard deviations and as proportions for categorical variables. Changes were evaluated using paired *t* test or Wilcoxon's test. Statistical significance was defined as $p < 0.05$.

Results

Baseline characteristics

The mean age at transplant was 53.6 ± 13.8 yr, and the mean time from transplant to conversion was 40.4 ± 23.4 months, with a range between 14 and 75 months post-transplant. Twelve patients were under CsA treatment and four under Tac treatment. All patients received MMF 1 g/d and methylprednisolone 4 mg/d. At baseline before conversion, the mean total daily CsA and Tac dose was 125.8 ± 18.8 mg/d and 2.0 ± 1.1 mg/d, respectively. The mean baseline values of S-Cr and eGFR were 1.22 ± 0.21 mg/dL and 46.0 ± 5.7 mL/min/1.73 m^2, respectively. Other clinical characteristics of the participants at baseline are reported in Table 1.

Table 1. Patient characteristics

Patient number	16
CNI	CsA: 12, Tac: 4
Gender (male/female)	11/5
HD duration (months)	56.1 ± 47.4
Age at transplant (yr)	53.6 ± 13.8
Post-transplant duration (months)	40.4 ± 23.4
Cause of end-stage renal disease	CGN; 5, IgA N; 2, renal sclerosis; 2, DM N; 2, hypoplastic kidney; 1, unknown; 4
Donor age (yr)	57.1 ± 6.8
Donor relation	Spouse 12, parent/child 4
Initial anti-A/B antibody titer	IgG; ×2–×4096 IgM; ×2–×512
S-Cr (mg/dL)	1.22 ± 0.21
eGFR (mL/min/1.73 m^2)	46.0 ± 5.7
Urinary albumin excretion (mg/g Cr)	16.6 ± 13.5
T-CHO (mg/dL)	205.3 ± 28.9
TG (mg/dL)	122.4 ± 44.2
HDL (mg/dL)	62.9 ± 16.1
LDL (mg/dL)	110.3 ± 18.1
CD19 (%)	2.6 ± 5.0
CD20 (%)	3.6 ± 5.4
IgG (mg/dL)	1002.5 ± 238.2
IgA (mg/dL)	217.9 ± 157.1
IgM (mg/dL)	61.4 ± 41.4
Anti-A/B antibody titer before conversion	IgG; ×1–×32 IgM; ×1–×4

Results are expressed mean ± SD. CNI, calcineurin inhibitor; CsA, cyclosporin A; Tac, tacrolimus; HD, hemodialysis; CGN, chronic glomerulonephritis; IgA N, IgA nehropahy; DM N, diabetes mellitus nephropathy; S-Cr, serum creatinine; eGFR, estimated glomerular filtration rate; T-CHO, total cholesterol; TG, triglycerides; HDL, high density lipoprotein cholesterol; LDL, low density lipoprotein cholesterol.

Change in doses and trough concentrations of everolimus and CNIs

The mean everolimus dose in the CsA group and Tac group was 1.71 ± 0.45 and 2.38 ± 0.75 mg/d, respectively, at month 1, 1.71 ± 0.45 and 2.38 ± 0.75 mg/d, respectively, at month 2, and 1.71 ± 0.45 and 2.25 ± 0.87 mg/d, respectively, at month 3. The mean everolimus concentration in the CsA group and Tac group was 4.72 ± 1.49 and 7.42 ± 2.84 ng/mL, respectively, at week 1, 5.04 ± 1.12 and 6.64 ± 0.93 ng/mL, respectively, at month 1, 4.56 ± 0.88 and 7.34 ± 1.57 ng/mL, respectively, at month 2, and 6.07 ± 3.24 and 5.96 ± 1.64 ng/mL, respectively, at month 3. In the CsA group, the mean CsA trough concentration was 104.2 ± 13.3 ng/mL at baseline compared with 34.4 ± 6.8 ng/mL at month 3, a reduction of 67%. The mean Tac trough concentration decreased by 58% from 3.9 ± 0.1 ng/mL at baseline to 2.25 ± 0.1 ng/mL at month 3.

Histology and C4d staining between baseline and three months after conversion

Histologic findings revealed no acute cellular rejection and no antibody-mediated rejection in all the patients at baseline. When the C4d staining patterns on the frozen sections were investigated, no patients showed diffuse $C4d^+$ staining. Focal $C4d^+$ staining and C4d-negative in the allograft biopsies were seen in three and 13 patients, respectively. Because focal and negative C4d staining was

diagnosed as "C4d$^-$," there was no C4d deposition in the allograft biopsies of all the recipients at baseline.

At three months after everolimus conversion, there were no acute cellular and antibody-mediated rejections in the allograft biopsies. C4d staining patterns showed no diffuse C4d$^+$ staining. Conversion from MMF to everolimus did not induce C4d deposition along peritubular capillaries in all of the graft biopsies (Table 2).

Change in peripheral blood CD19$^+$ and CD20$^+$ and plasma immunoglobulin

The mean peripheral blood CD19$^+$ cells and CD20$^+$ cells was 2.6 ± 5.0% and 3.6 ± 5.4%, respectively, at baseline, 2.7 ± 3.9% and 2.8 ± 4.1%, respectively, at month 1, 3.3 ± 5.3% and 3.1 ± 2.7%, respectively, at month 2, and 3.0 ± 4.7% and 4.0 ± 5.6% at month 3, and there were no significant changes in peripheral blood CD19$^+$ and CD20$^+$ cells during the study period. The mean serum IgG and IgM levels was 1002.5 ± 238.2 and 61.4 ± 41.4 mg/dL, respectively, at baseline, 1035.8 ± 210.8 and 61.8 ± 37.7 mg/dL, respectively, at month 1, 1036.5 ± 287.6 and 64.2 ± 38.4 mg/dL, respectively, at month 2, and 1048.6 ± 359.4 and 63.9 ± 40.3 mg/dL, respectively, at month 3, and there were significant changes in plasma immunoglobulin (IgG and IgM) during the study period.

Table 2. C4d staining

	Before conversion	Three months after conversion
C4d staining (−)	13	14
Focal C4d staining (+)	3	2
Diffuse C4d staining (+)	0	0

Change in anti-A/B antibody titers

Conversion from MMF to everolimus resulted in a significant elevation of anti-A/B IgG antibody titer from 7.4 (×1–×32) at baseline to 11.3 (×1–×64) at month 1, 19.3 (×1–×128) at month 2, and 11.9 (×1–×64) at month 3. However, the elevation of anti-A/B IgG antibody titer was only less than four times. There was no significant change in anti-A/B IgM antibody titer during the study period (Fig. 1).

Renal function

The mean S-Cr levels significantly decreased from 1.22 ± 0.21 mg/dL at baseline to 1.10 ± 0.19 mg/dL at month 1, 1.13 ± 0.20 mg/dL at month 2, and 1.12 ± 0.21 mg/dL at month 3. The mean eGFR value significantly increased from 46.0 ± 5.7 mL/min 1.73 m^2 at baseline to 51.8 ± 7.5 mL/min 1.73 m^2 at month 1, 49.1 ± 7.0 mL/min 1.73 m^2 at month 2, and 50.7 ± 7.8 mL/min 1.73 m^2 at month 3 (Fig. 2).

Albuminuria

The average urinary albumin excretion significantly increased after conversion. Baseline albuminuria was 16.6 ± 13.5 mg/g Cr, increasing to 32.4 ± 33.6 mg/g Cr at month 1, 30.4 ± 18.7 mg/g Cr at month 2, and 34.8 ± 20.0 mg/g Cr at

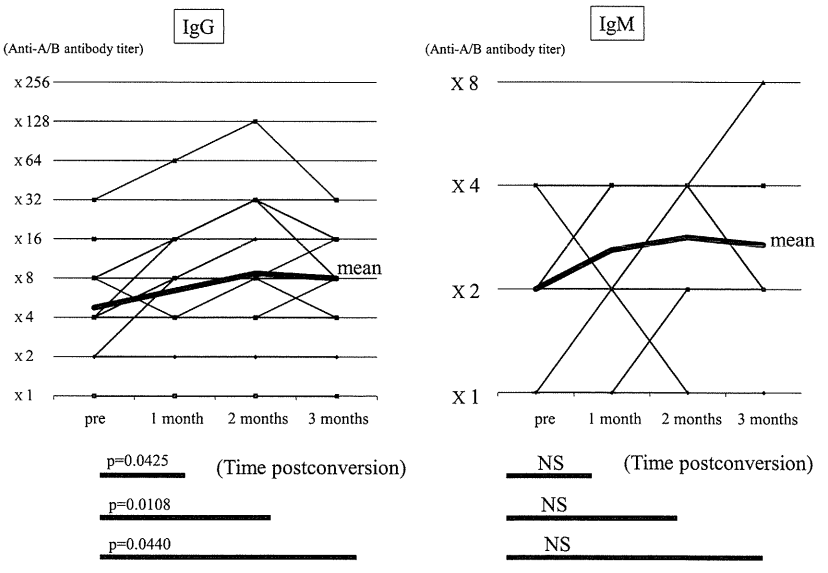

Fig. 1. Changes in anti-A/B antibody titers (IgG and IgM) during the study period. Heavy lines represent the mean of antibody titers.

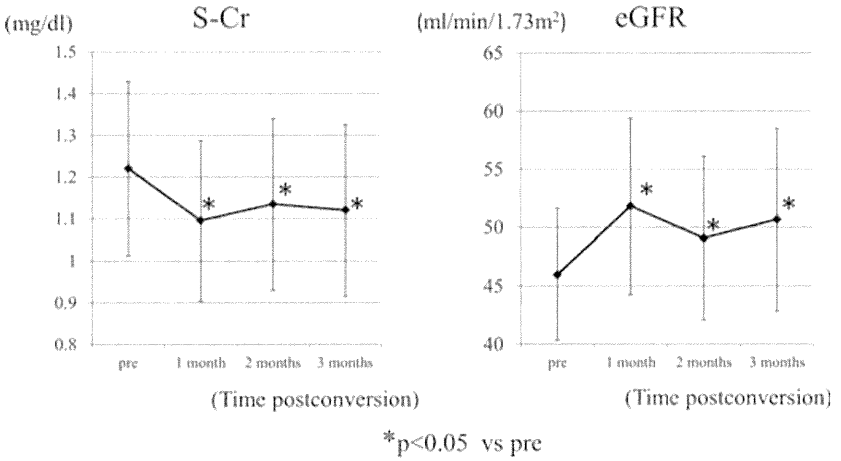

Fig. 2. Effect of conversion to everolimus on renal function.

month 3. Among the 16 recipients, six required angiotensin II receptor blocker (ARB) administration due to the elevation of urinary albumin excretion. We considered the initiation of ARB administration at more than 50 mg/g Cr, and ARB administration was started at a mean of 52.3 ± 12.4 d after conversion. Urinary albumin excretion was slightly and significantly elevated after conversion to everolimus. However, the average urinary albumin excretion was in the range of microalbuminuria (the lower limit of microalbuminuria) by the administration of ARB (Fig. 3).

Safety

There were no events leading to discontinuation of everolimus or reconversion to MMF, and no death and graft loss occurred during the study. Adverse events were categorized as drug related when they occurred after conversion, the most frequent of which were hypercholesterolemia (75%), peripheral edema (50%), albuminuria (37.5%), aphthous stomatitis (37.5%), transient general fatigue (37.5%), anemia (12.5%), and acne (6.25%).

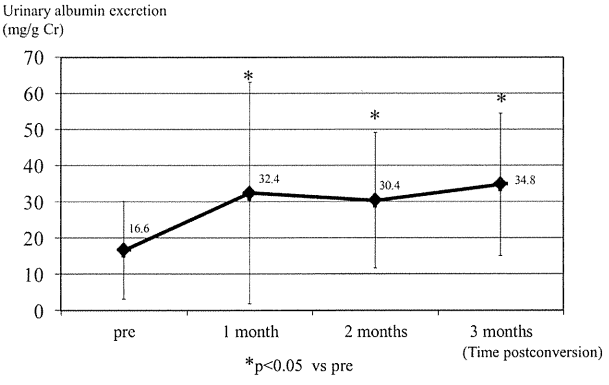

Fig. 3. Effect of conversion to everolimus on urinary albumin excretion.

Among the 16 recipients, 12 required initiation of statins or change to strong statins due to hypercholesterolemia. The administration of statins was useful in reducing total cholesterol values. Peripheral edema was treated with loop diuretics and was controllable. Aphthous stomatitis and acne responded to care of the oral cavity and skin and local application of corticosteroids. The patients with anemia were given an erythropoiesis-stimulating agent.

Discussion

In this study, stable ABO-incompatible kidney transplant recipients were converted from MMF with standard exposure CNIs to everolimus with very low exposure CNIs. Conversion to everolimus with CNI minimization elicited no acute cellular rejection and no C4d deposition along peritubular capillaries and maintained suppressed peripheral blood $CD19^+$ and $CD20^+$ cells and plasma immunoglobulin levels in the ABO-incompatible kidney transplant recipients for at least three months after conversion. These results demonstrated that short-term conversion from MMF to everolimus might be a safe and effective alternative for ABO-incompatible kidney transplant recipients requiring temporary discontinuation of MMF. To our knowledge, this is the first demonstration of everolimus application in ABO-incompatible kidney transplantation.

A slight elevation (less than four times) of anti-A/B antibody titer (IgG) occurred in our present study. Antibody-mediated rejection due to ABO histo-blood antigens occurs within one month after transplantation, with a particularly high frequency within the first week, which is considered the "critical period" of antibody-mediated rejection, after which "accommodation" is subsequently established. Once accommodation

is established, there are no further instances of antibody-mediated rejection due to anti-A/B antibodies, and this continues for the life of the graft (2). All recipients were enrolled in this study at least one yr after transplantation, and accommodation was already established in all cases. Although there have been no reports that acute antibody-mediated rejection is caused by less than four times elevation of anti-A/B antibody titer in ABO-incompatible kidney transplant recipients and conversion to everolimus did not induce C4d deposition along peritubular capillaries in all of the graft biopsies in this study, introduction of everolimus for de novo ABO-incompatible kidney transplantation or conversion from MMF during the early phase of post-transplantation before accommodation is established may induce antibody-mediated rejection due to anti-A/B antibodies.

Our study demonstrated that conversion to everolimus with CNI minimization at a mean of 3.3 yr after kidney transplantation decreased S-Cr levels and increased eGFR values, significantly, in the short term. The ASCERTAIN study revealed that in kidney transplant recipients who were, on average, five yr post-transplant, introduction of everolimus with elimination of CNI or a marked reduction of CNI had no overall benefit on renal function and was associated with more frequent adverse events and discontinuations. However, they identified that patients with a creatinine clearance of more than 50 mL/min might benefit from a change in therapy more than six months after transplantation (14). Patients enrolled in our study had relatively good graft function (eGFR 46.0 $\pm$ 5.7 mL/min/1.73 m^2 at baseline). They had properly received a living donor kidney, and their transplanted kidney was considered to maintain functional reserve. Our study showed that short-term conversion of selected ABO-incompatible kidney transplant recipients with good renal function from MMF to everolimus did not seem to induce a deterioration of graft function even at a late post-transplant stage.

Urinary albumin excretion was elevated after conversion to everolimus in this study. Problems can be posed by albuminuria or proteinuria associated with the use of mTOR inhibitors. A high rate of albuminuria has been reported in transplant recipients receiving mTOR inhibitors (everolimus and sirolimus) (15). In renal transplant recipients, microalbuminuria predicts graft loss and all-cause mortality (16–18). This present study demonstrated that everolimus-induced urinary albumin excretion was reduced to the range of microalbuminuria (the lower limit of microalbuminuria) by administration of ARB in the short-term after conversion. Previous reports have shown that damaged grafts may leak more urine protein and albumin when treated with mTOR inhibitors. The conversion to everolimus, as with sirolimus, is not advised in patients with proteinuria >800 mg/d (19). On the other hand, most reports of de novo use or early conversion trials have not shown a significant increase in proteinuria (11, 20). Our results showed that elevation of urinary albumin excretion due to short-term conversion to everolimus was controllable by ARB administration, when there was little damage to the transplanted graft. However, increased albuminuria may well have an unfavorable effect for long-term graft and patient survival.

The profile of adverse events was consistent with that reported previously for mTOR inhibitors (21). The adverse events that occurred after conversion to everolimus were hypercholesterolemia, peripheral edema, albuminuria, aphthous stomatitis, transient general fatigue, anemia, and acne. In accordance with previous reports (11, 12), the patients were switched from MMF with standard exposure CNIs to everolimus with very low exposure CNIs. It may be valuable to use the other protocol such as a lower dose of everolimus with standard exposure CNIs. No patients undergoing treatment for any adverse effects required discontinuation of everolimus during the study period. Several adverse effects due to temporary conversion to everolimus remained within acceptable limits. However, they may induce discontinuation in the long term. In fact, it was recently reported that the adverse effects of mTOR inhibitors accounted for the 20–40% drop-out rate in a clinical Phase III trial (21). Long-term follow-ups are needed to evaluate the safety and tolerability of everolimus in ABO-incompatible kidney transplantation.

ABO-incompatible kidney transplantation is an immunologically high-risk procedure and powerful immunosuppressant drug combinations including rituximab and MMF are needed for desensitization (3–7). Desensitization protocols for ABO-incompatible kidney transplantation, consisting of rituximab and MMF, may induce several adverse effects such as CMV infection. Previously, we demonstrated that the incidence of CMV reactivation by CMV antigenemia was 48% in ABO-incompatible kidney transplant recipients at our institution (3). There may be cases in which the discontinuation of MMF may be required for a short period due to intractable CMV infection in ABO-incompatible kidney transplantation. CMV replication is dependent upon one of two mTOR pathways, and *in vitro* studies have supported an association between mTOR inhibitors and

decreased CMV (22). Recently, a pooled analysis from several studies confirmed a significant reduction in the incidence of CMV infection and viremia in everolimus-treated patients compared with MMF-treated patients (23). In such cases, everolimus may therefore be a potentially effective immunosuppressant for ABO-incompatible kidney transplantation.

The present study might have limitations because of the small sample size and very short duration of the study. We have only 16 patients due to limited number of patients. We could not perform a randomized and control study although it is ideal. Although the present study design might not be perfect, all of the recipients enrolled in this study received graft biopsies before and after termination of the study. Our results indicated that safety of short-term conversion of stable ABO-incompatible kidney transplant recipients from MMF with standard exposure CNIs to everolimus with very low exposure CNIs.

In conclusion, everolimus conversion with CNI minimization was achieved in ABO-incompatible kidney transplant recipients for three months in our present study. Conversion to everolimus with CNI minimization elicited no acute rejection and no C4d deposition and suppressed B cell function and activation for at least three months after conversion. In cases such as intractable CMV infection, temporary conversion to everolimus may therefore be a safe and effective alternative in ABO-incompatible kidney transplantation. To assess the effectiveness of everolimus for ABO-incompatible kidney transplantation in the long term, further prospective well-controlled, long-term follow-up trials with a larger number of patients are needed in future.

Authors' contributions

We would like to note that among the authors, J. Uchida, Y. Machida, N. Kuwabara, K. Kabei, and T. Nakatani designed the study, and J. Uchida and T. Nakatani wrote the paper. J. Uchida, Y. Machida, N. Kuwabara, and K. Kabei participated in the patients' follow-up and collected data, whereas T. Naganuma, H. Kawashima, T. Iwai, and N. Kumada analyzed the data.

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