enzyme inhibitor/angiotensin II receptor blocker (ACE-I/ARB) [2–4]. Impact of PTA on mortality and graft failure in posttransplant patients has been clearly demonstrated by Molnar [5], who has shown higher rates of mortality and graft failure in groups of patients whose hemoglobin (Hb) levels were lower than 11.0 g/dl. Administration of recombinant human erythropoietin (rHuEPO-ad) has been shown to be effective in correcting PTA [6]. In our previous study, we showed that elevation of hematocrit (Ht) from 31.7 ± 1.9% to 33.6 ± 3.6% by rHuEPO-ad in posttransplant patients improved left-ventricular hypertrophy [6]. An identical conclusion was also reported by Hayashi [7], and these results may represent the potential benefit of rHuEPO-ad on the cardiovascular system in posttransplant patients. In the same study, we investigated the impact of rHuEPO-ad on QOL of posttransplant patients, scored by the 36-item Short Form (SF-36) [8], an international questionnaire for analysis of QOL. To our surprise, rHuEPO-ad failed to improve QOL in posttransplant patients. This opposes the established beneficial role of rHuEPO-ad on QOL in subjects with cancer or chronic renal failure [9]. Apparently, a possible explanation for our negative result is that the targeted level of Ht was not sufficient to improve QOL. Accordingly, the aim of this study was to test whether improvement of Hb to 13.3 g/dl by rHuEPO-ad can improve QOL in posttransplant patients.

Patients, materials, and methods

This was a prospective cohort study on patients recruited over a 2-month period starting from 1 September 2003, which included a follow-up period over 6 months. The subjects were patients who met the following inclusion criteria: (1) having undergone renal transplantation at the Department of Urology of Osaka University Medical School and subsequently followed up at Inoue Hospital, (2) without serious rejection, defined by resistance to the conventional treatments and progressive loss of renal function, (3) with Hb level less than 12.0 g/dl with or without administration of erythropoietin, (4) with either an iron saturation level over 16% or a serum ferritin level over 50 ng/ml, and (5) who consented to participate in the study. The following patients were excluded: (1) those who were hypersensitive to erythropoietin, (2) those who had a history of gelatin allergy, (3) women who were pregnant, breast feeding or had child-bearing potential, (4) those with severe heart failure, (5) those with nephrotic syndrome or severe edema, (6) those with severe hypertension that was resistant to antihypertensive treatments, (7) those with a history of myocardial or cerebral infarction, or pulmonary embolism, (8) those with certain predispositions such as drug allergies, (9) those with serious complications such as pancytopenia, malignant neoplasms, systemic amyloidosis, severe infections or severe hemorrhagic lesions, and (10) those using antipsychotic drugs or with dementia. The endpoint of the present study was QOL. A self-reported questionnaire survey (version 2.0 of the Japanese edition [10, 11] of SF-36) was used to score the QOL before and 3 and 6 months after administration of rHuEPO. The present study was conducted according to Declaration of Helsinki principles and was approved by the local ethical committee of our institution. All patients provided written informed consent prior to participation.

Treatment

rHuEPO preparation Epoetin-α (Kyowa Hakko Kirin Co., Ltd, Tokyo, Japan) was administered subcutaneously at dose of 6,000 IU once a week in order to increase Hb level to 13.3 g/dl. For safety, increase of Hb was maintained below 0.3 g/dl per week. Once Hb reached 13.3 g/dl, administration of rHuEPO was adjusted within the range of 6,000–12,000 IU once every 2 weeks or 6,000 IU once a week, as required.

Observations and tests

QOL was evaluated at the start of follow-up, and at 3 and 6 months after the commencement of the study, and the changes in the eight subscales of SF-36 [physical function (PF), limitations due to physical problems (RP), bodily pain (BP), general health perceptions (GH), vitality (VT), social function (SF), limitations due to emotional problems (RE), and mental health (MH)] were obtained to calculate the physical and mental summary score (PSC and MSC) to evaluate improvement of QOL by administration of rHuEPO. Hemoglobin (Hb) and serum creatinine (sCr) were also measured and eGFR was calculated at time of QOL evaluation. sCr was measured by enzymatic method, and GFR-estimating equations for Japanese eGFR, i.e., eGFR [ml/(min 1.73 m^2)] = 194 × $sCr^{-1.094}$ × $age^{-0.287}$ × 0.739 (if female), were applied to calculate eGFR in this study [12]. Differences in laboratory findings were tested by one-way analysis of variance (ANOVA) (repeated). r^2 values given by correlation analysis of two factors were converted to P value according to sample numbers. Results are expressed as mean ± standard error on the mean (SEM) and $P < 0.05$ was considered significant. All analyses were performed using SAS software version 6.12.

Results

Twenty-four patients were recruited and analyzed in the 2 months from 1 September to 31 October in 2003. The

patients included 12 men and 12 women with mean age of 36.6 ± 2.2 years (27–59 years) and whose mean time since renal transplantation was 7.1 ± 0.8 years (5.0–20 years). Mean Hb before administration of rHuEPO (rHuEPO-ad) was 10.5 ± 0.2 g/dl and improved significantly to 13.7 ± 0.6 g/dl after 3 months of rHuEPO-ad ($P < 0.01$). Thereafter, values were maintained throughout the rest of the observation period (6 months: 13.7 ± 0.3 g/dl; $P < 0.01$ versus before, NS versus 3 months). Mean serum creatinine (sCr) and eGFR before rHuEPO-ad were 1.30 ± 0.07 mg/dl and 48.5 ± 2.7 ml/(min 1.73m^2), respectively, and were not affected by rHuEPO-ad (Table 1). Among the eight subscales of SF-36, posttransplant patients had preserved body pain (BP: 49.3 ± 2.4), vitality (VT: 52.4 ± 2.6), general health perception (GH: 47.5 ± 1.4), and mental health (MH: 48.5 ± 2.8), but impaired physical function (PF: 45.3 ± 2.6), limitations due to physical problem (RP: 30.7 ± 4.0), limitations due to emotional problems (RE: 34.6 ± 4.3), and social function (SF: 39.8 ± 3.1) (Fig. 1). The summary scores calculated from these eight subscales showed preserved mental summary score (MSC: 54.1 ± 2.3) but impaired physical summary score (PSC: 32.6 ± 3.2) (Fig. 2). No correlation was found between initial Hb and initial PSC or MSC (data not shown). Three months of rHuEPO-ad improved PCS, accompanied by improvement of PF, RP, SF, and RE (Figs. 1, 2). Thereafter, scores were maintained throughout the rest of the observation period. There were no correlations between initial PSC and initial MSC, or between changes in PSC and MSC with rHuEPO-ad, which demonstrates the independence of these two parameters (Fig. 3). Interestingly, inverse correlation was observed between initial PSC or MSC and their responses to rHuEPO-ad for 6 months (Fig. 4). Accordingly, patients whose initial PSC was lower than 39.6 or whose initial MSC was lower than 39.4 were expected to improve their PSC or MSC by more than 10 after rHuEPO-ad. When patients were divided into two subgroups (group A: initial PSC > 39.6, $n = 8$; group B: initial PSC ≤ 39.6, $n = 16$), rHuEPO-ad significantly improved PSC in group B, whereas it failed to show any impact in group A (Fig. 5a). Only two patients had MSC lower than 39.4, which was not sufficient for further analysis (Fig. 5b).

Discussion

We investigated the effect on QOL of increase of Hb over 13.3 g/dl by rHuEPO-ad in anemic posttransplant patients. As shown in Table 1, we successfully improved Hb to over 13.3 g/dl by rHuEPO-ad in this study. Although the initial level of Ht did not correlate with initial QOL in agreement with the previous study [13], significant improvements of physical QOL were observed after increase of Hb over 13.3 g/dl. No relationships were observed among magnitude of correction of Hb and degree of improvement of PSC or MSC. The clear contrast between the present study, which succeeded in improving QOL by rHuEPO-ad, and our previous study, which failed to improve QOL by rHuEPO-ad [6], demonstrates the importance of the target level of Hb for QOL in posttransplant patients. In our previous study, target level of Ht (33–36%, corresponding to Hb of 11.0–12.0 g/dl) was determined on the basis of The National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NKF-K/DOQI) recommendation and related factors [14], but this recommendation is developed for chronic renal failure subjects including those on hemodialysis and may not be sufficient to improve QOL of transplant patients, probably due to the positive impact of renal transplantation itself on QOL [15]. This idea is supported by the study of Ogutmen [16], which showed better QOL in transplant patients than in hemodialysis or peritoneal dialysis patients.

Although the present study demonstrated the positive impact of rHuEPO-ad on QOL, negative aspects of this treatment have also been reported in recent studies. Accordingly, in patients with chemotherapy-induced anemia, rHuEPO-ad increases risk of thromboembolic events and hypertension [9]. rHuEPO-ad also increases risk of cardiovascular events in anemic subjects with renal insufficiency [17]. We did not record any vascular embolic side-effects in the present study. We also did not record adverse effects on renal function (Table 1). Furthermore, it has been speculated from recent study that cardiovascular side-effects of rHuEPO-ad might be specific to subjects who are resistant to rHuEPO therapy [18]. However, we cannot neglect the potential risk of vascular embolism due to the higher target level of Hb. One strategy to minimize this risk is

Table 1 Hemoglobin, serum creatinine, and eGFR

Total ($n = 24$)	Baseline (BL)	After 3 months (3 M)	After 6 months (6 M)	P value		
				BL versus 3 M	BL versus 6 M	3 M versus 6 M
Hb (g/dl)	10.5 ± 0.2	13.7 ± 0.6	13.7 ± 0.3	$P < 0.01$	$P < 0.01$	NS
sCr (mg/dl)	1.30 ± 0.07	1.28 ± 0.07	1.27 ± 0.07	NS (by ANOVA)		
eGFR [ml/(min 1.73 m^2)]	48.5 ± 2.7	49.1 ± 2.5	49.7 ± 2.6	NS (by ANOVA)		

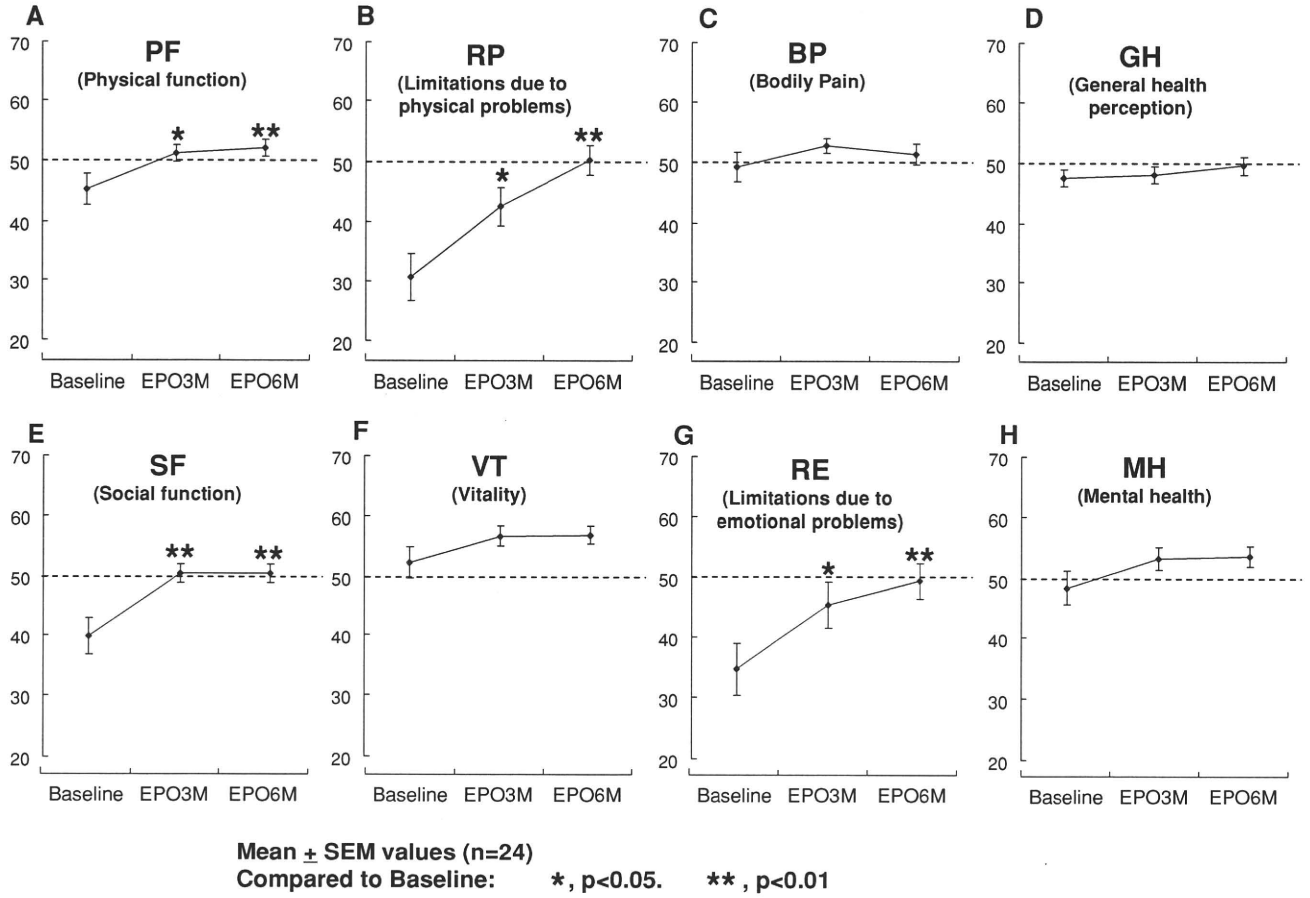

Fig. 1 The eight subscales of SF-36 in posttransplant patients before and after administration of rHuEPO. **a** physical function (PF), **b** limitations due to physical problem (RP), **c** body pain (BP), **d** general health perception (GH), **e** social function (SF), **f** vitality (VT), **g** limitations due to emotional problems (RE), and **h** mental health (MH)

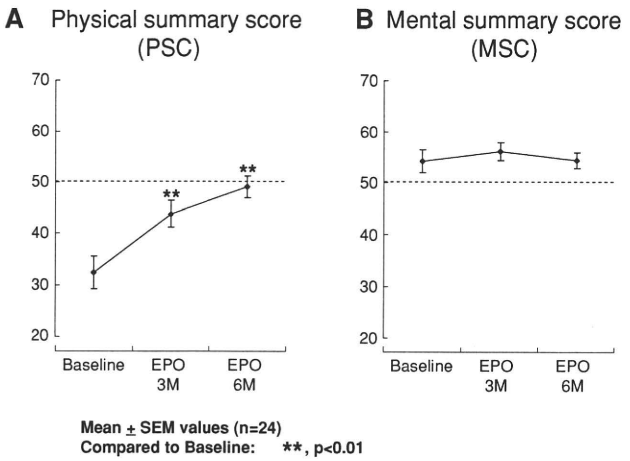

Fig. 2 Calculated physical (**a**) and mental summary scores (**b**) in posttransplant patients before and after administration of rHuEPO

identification of the groups of subjects whose QOL may or may not be improved by rHuEPO-ad. If rHuEPO-ad could be applied only to QOL responders, this would not only reduce the risk of side-effects due to unnecessary rHuEPO-ad, but would also have a beneficial impact on medication cost. In the present study, we identified that improvement of QOL by rHuEPO-ad is highly dependent on initial level of QOL before rHuEPO-ad (Fig. 4). The correlation study indicated that rHuEPO-ad is expected to improve PSC or MSC by more than 10 when initial PSC is lower than 39.6 or initial MSC is lower than 39.4. Indeed, the group of patients whose initial PSC was lower than 39.6 showed significant increase in PSC with rHuEPO-ad, whereas those who had initial PSC higher than 39.6 failed to show improvement of PSC with rHuEPO-ad. Although further investigations are necessary to conclude the exact values of initial PSC and MSC that can predict QOL responders, the present study firstly identifies the usefulness of QOL scoring of post-transplant subjects by SF-36 for the prediction of QOL responders with rHuEPO-ad. The limitation of this study is the small number of samples ($n = 24$) and the short period of observation (6 months), and further investigations are necessary to conclude the impact of rHuEPO-ad on QOL and safety of rHuEPO-ad in posttransplant patients.

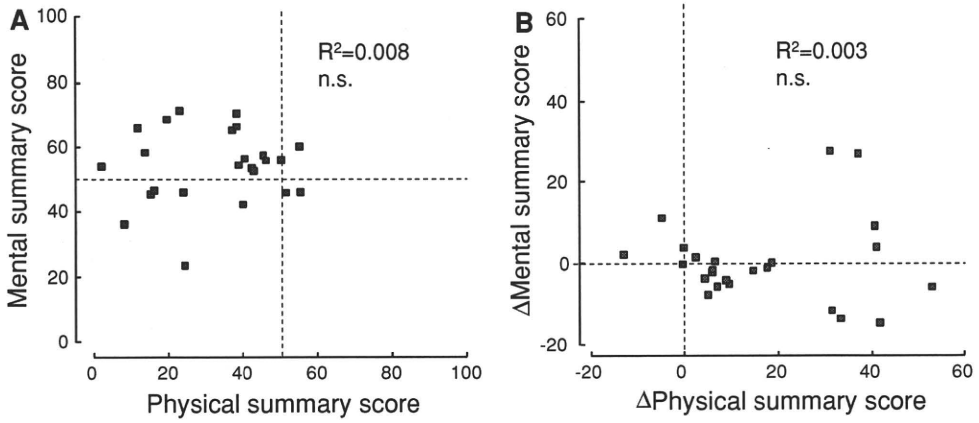

Fig. 3 No correlation between initial physical and initial mental summary score (**a**) or between changes in physical and mental summary score (**b**) with administration of rHuEPO

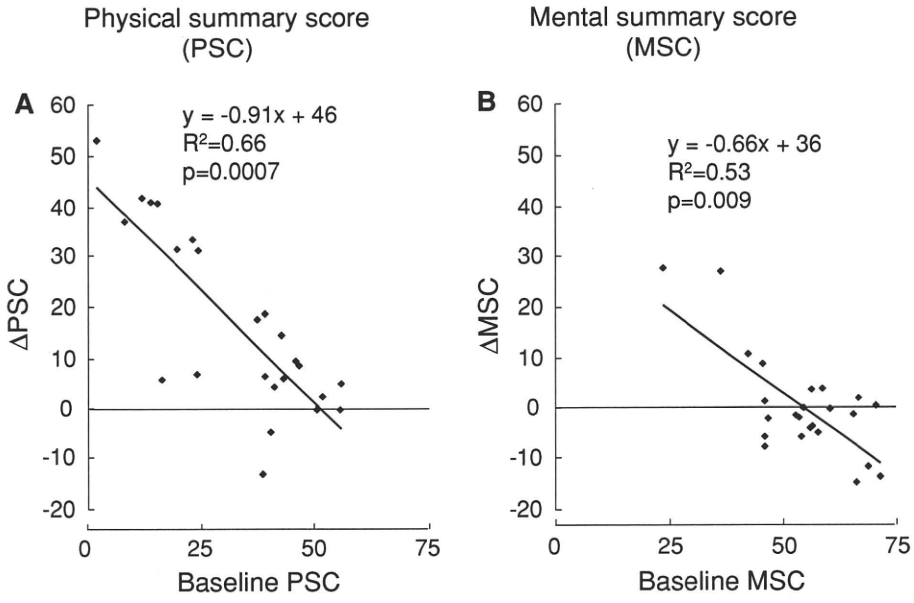

Fig. 4 **a** Correlation between physical summary score and change in physical summary score with administration of rHuEPO. **b** Correlation between mental summary score and the change in mental summary score with administration of rHuEPO

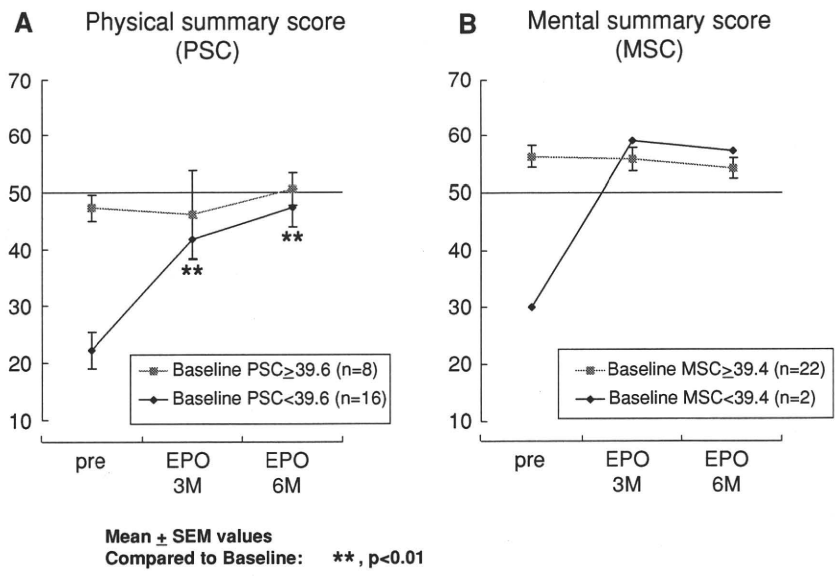

Fig. 5 Calculated physical and mental summary score in posttransplant patients before and after administration of rHuEPO. **a** Physical summary score in groups of patients whose initial physical summary score was over 39.6 ($n = 8$) or under 39.6 ($n = 16$). **b** Mental summary score in groups of patients whose initial mental summary score was over 39.4 ($n = 22$) or under 39.4 ($n = 2$)

In conclusion, anemia in posttransplant patients has negative impacts on their QOL. Scoring of posttransplant patient's QOL by SF-36 is useful to identify the group of patients whose QOL will be improved by administration of rHuEPO. Posttransplant anemic patients whose initial PSC was lower than 39.6 or whose initial MSC was lower than 39.4 are strongly expected to experience improved QOL by targeting Hb over 13.3 g/dl by administration of rHuEPO.

Acknowledgments The authors would like to thank all of the kidney recipients who took part in this study. We also express our sincere appreciation to Dr. Shun-ichi Fukuhara for giving us permission to use SF-36. We also thank Ms. Kakuko Yamamoto, Ms. Miyoko Inoue, Mr. Hiroshi Kobayashi, Mr. Hideki Kitada, and Ms. Miyoko Yamauchi for their assistance in the study.

References

1. Zadrazil J, Horák P, Horcicka V, Zahálková J, Strébl P, Hrubý M. Endogenous erythropoietin levels and anemia in long-term renal transplant recipients. Kidney Blood Press Res. 2007;30:108–16.
2. Sinnamon KT, Courtney AE, Maxwell AP, McNamee PT, Savage G, Fogarty DG. Level of renal function and serum erythropoietin levels independently predict anaemia post-renal transplantation. Nephrol Dial Transplant. 2007;22:1969–73.
3. Shah N, Al-Khoury S, Afzali B, Covic A, Roche A, Marsh J, et al. Posttransplantation anemia in adult renal allograft recipients: prevalence and predictors. Transplantation. 2006;8:1112–8.
4. Montanaro D. Anemia after renal transplantation. G Ital Nefrol. 2007;24:13–22.
5. Molnar MZ, Czira M, Ambrus C, Szeifert L, Szentkiralyi A, Beko G, et al. Anemia is associated with mortality in kidney-transplanted patients—a prospective cohort study. Am J Transplant. 2007;7:818–24.
6. Kawaguchi T, Moriyama T, Suzuki K, Hatori M, Tanaka T, Takahara S, et al. Pilot study of the optimum hematocrit for patients in the predialysis stage after renal transplantation. Transplant Proc. 2004;36:1293–6.
7. Hayashi T, Suzuki A, Shoji T, Togawa M, Okada N, Tsubakihara Y, et al. Cardiovascular effect of normalizing the hematocrit level during erythropoietin therapy in predialysis patients with chronic renal failure. Am J Kidney Dis. 2000;35:250–6.
8. Ware JE Jr, Sherbourne CD. The MOS 36-item short-form health survey (SF-36). I. Conceptual framework and item selection. Med Care. 1992;30:473–83.
9. Bokemeyer C, Aapro MS, Courdi A, Foubert J, Link H, Osterborg A, et al. European Organisation for Research and Treatment of Cancer (EORTC) Taskforce for the elderly. EORTC guidelines for the use of erythropoietic proteins in anaemic patients with cancer: 2006 update. Eur J Cancer. 2007;43:258–70.
10. Fukuhara S, Bito S, Green J, Hsiao A, Kurokawa K. Translation, adaptation, and validation of the SF-36 Health Survey for use in Japan. J Clin Epidemiol. 1998;51:1037–44.
11. Fukuhara S, Ware JE Jr, Kosinski M, Wada S, Gandek B. Psychometric and clinical tests of validity of the Japanese SF-36 Health Survey. J Clin Epidemiol. 1998;51:1045–53.
12. Matsuo S, Imai E, Horio M, Yasuda Y, Tomita K, Nitta K, Yamagata K, Tomino Y, Yokoyama H, Hishida A. Revised equations for estimating glomerular filtration rate (GFR) from serum creatinine in Japan. Am J Kidney Dis 2009 (in press).
13. Shah VS, Ananth A, Sohal GK, Bertges-Yost W, Eshelman A, Parasuraman RK, et al. Quality of life and psychosocial factors in renal transplant recipients. Transplant Proc. 2006;38:1283–5.
14. National Kidney Foundation. IV. NKF-K/DOQI clinical practice guidelines for anemia of chronic kidney disease: update 2000. Am J Kidney Dis. 2001;37:S182–238.
15. Fiebiger W, Mitterbauer C, Oberbauer R. Health-related quality of life outcomes after kidney transplantation. Health Qual Life Outcomes. 2004;2:2.
16. Ogutmen B, Yildirim A, Sever MS, Bozfakioglu S, Ataman R, Erek E, et al. Health-related quality of life after kidney transplantation in comparison intermittent hemodialysis, peritoneal dialysis, and normal controls. Transplant Proc. 2006;38:419–21.
17. Singh AK, Szczech L, Tang KL, Barnhart H, Sapp S, Wolfson M, et al. Correction of anemia with epoetin alfa in chronic kidney disease. N Eng J Med. 2006;355:2085–98.
18. Kilpatrick RD, Critchlow CW, Fishbane S, Besarab A, Stehman-Breen C, Krishnan M, et al. Greater epoetin alfa responsiveness is associated with improved survival in hemodialysis patients. Clin J Am Soc Nephrol. 2008;3:1077–83.

Journal of Medical Microbiology (2009), **58**, 1149–1153 DOI 10.1099/jmm.0.010934-0

Assessment of East Asian-type *cagA*-positive *Helicobacter pylori* using stool specimens from asymptomatic healthy Japanese individuals

Itaru Hirai,[1] Tadahiro Sasaki,[1] Ai Kimoto,[1] Saori Fujimoto,[1] Toshiki Moriyama[2] and Yoshimasa Yamamoto[1]

[1]Department of Bioinformatics, Osaka University Graduate School of Medicine, Osaka 565-0871, Japan

[2]Health Care Center, Osaka University, Osaka 560-0043, Japan

Correspondence
Itaru Hirai
hiraii@sahs.med.osaka-u.ac.jp

Received 9 March 2009
Accepted 11 May 2009

Recent investigations have suggested that CagA, a virulence factor of *Helicobacter pylori* and known to have multiple genotypes, plays a critical role in the development of stomach cancer. However, the prevalence of *cagA*-positive *H. pylori* strains and the *cagA* genotypes have not been well studied in healthy individuals because of the difficulty in collecting gastric specimens. In the present study, we assessed the prevalence of infection with *H. pylori*, particularly the strains with the East Asian *cagA* genotype (which is more potent in causing gastric diseases), among healthy asymptomatic Japanese individuals by a noninvasive method using stool specimens. The *H. pylori* antigen was detected in 40.3 % of healthy asymptomatic adult individuals (n=186) enrolled in the study. For the detection and genotyping of the *cagA* gene, DNA was extracted from the stool specimens of these individuals and analysed by PCR. We detected the East Asian *cagA* genotype in the DNA samples of a significantly high number (63.1 %) of healthy asymptomatic Japanese individuals. These results indicate that a significant number of asymptomatic healthy Japanese individuals were infected with highly virulent *H. pylori*.

INTRODUCTION

The significance of various virulence factors of *Helicobacter pylori* has been studied with regard to the pathology of gastric diseases, such as peptic ulcers and gastric cancer. Among these, CagA is one of the well-studied virulence factors of *H. pylori*. The CagA protein is classified into two major types – the East Asian type and the Western type – depending on the combination of the four domains (A, B, C and D) present on the variable region of the C-terminal domain of this protein (Higashi *et al.*, 2002a; Yamaoka *et al.*, 2000b). Each domain contains a single Glu-Pro-Ile-Tyr-Ala (EPIYA) motif (Covacci *et al.*, 1993). Recent studies have revealed that EPIYA motifs are potential targets of the Src family of protein tyrosine kinases. Furthermore, of the four EPIYA motifs in each domain, EPIYA-D of the East Asian CagA protein has a stronger transforming activity than that of the Western type because of the stimulation of signal transduction cascades (Higashi *et al.*, 2002b; Naito *et al.*, 2006). Therefore, the East Asian CagA is more virulent than the Western type, which contains the EPIYA-C domain. In addition, it has been reported that the East Asian CagA probably plays a more effective pathophysiological role than the Western type in the development of gastric diseases caused by *H. pylori* infection (Azuma, 2004). Therefore, extensive genotyping of the *cagA* gene has been carried out in many countries using *H. pylori* strains clinically isolated from patients with gastric diseases (Devi *et al.*, 2006; Kanada *et al.*, 2008; Vilaichone *et al.*, 2004; Yamaoka *et al.*, 2000b, 2008; Yamazaki *et al.*, 2005b).

In contrast, there have been only few studies on the different *cagA* genotypes of *H. pylori* in healthy asymptomatic individuals, mainly because of the difficulty in collecting gastric biopsy samples from healthy individuals (Chattopadhyay *et al.*, 2002; Molnar *et al.*, 2008; Yamaoka *et al.*, 2000a). Therefore, we established a genotyping method that involves the use of stool specimens, which were collected from healthy asymptomatic individuals (Hirai *et al.*, 2009). In the present study, we determined the incidence of *H. pylori* infection by using a noninvasive method to analyse stool specimens and detected the East Asian *cagA* genotype in healthy asymptomatic Japanese individuals.

METHODS

Participants and stool specimens. This study was conducted in Osaka, Japan, from June 2007 to October 2007. Initially, a total of 235 individuals were enrolled in this study. These individuals were screened for age (>39 years) and medical history. The exclusion

criteria included any antibiotic treatment in the past 3 months, eradication therapy for *H. pylori*, and a confirmed diagnosis of digestive tract diseases. Finally, 186 individuals (65 women, 121 men; age range 40–63 years) participated in this study. Stool specimens were collected from the participants, and they were also asked to fill out questionnaires. This study was approved by the ethics committee of the Osaka University Graduate School of Medicine, Osaka, Japan.

Detection of the *H. pylori* antigen and DNA extraction. The individuals' stool specimens were tested for catalase, i.e. an *H. pylori* antigen, by immunochromatographic analysis using a commercially available rapid test kit (TestMate Rapid Pylori Antigen; BD Japan), according to the manufacturer's instructions (Cardenas *et al.*, 2008; Suzuki *et al.*, 2002). The detection limit of this kit is 18.8 ng ml^{-1} of protein concentration (equivalent to 10^4–10^5 bacterial cells ml^{-1}). Bacterial DNA was extracted from stool specimens that tested positive for the *H. pylori* antigen using the QIAmp DNA stool mini kit (Qiagen) according to the manufacturer's instructions with the following minor modifications. Approximately 1 g of each stool specimen was suspended in 3.0 ml ASL buffer (supplied in the kit). After mixing the suspension, approximately 1.2 ml of the supernatant was used for DNA extraction. The extracted DNA was dissolved in 200 µl AE buffer (supplied in the kit) and stored at −20 °C until further use.

PCR analysis. All PCR analyses were performed according to methods described in a previous report (Hirai *et al.*, 2009). The sequences of the primers and the probe used in this study are shown in Table 1. For the template, we used a solution containing 50 ng DNA µl^{-1} or a 10-fold dilution of the DNA sample (approx. 5–10 ng µl^{-1}), which minimized the effect of the inhibitors possibly present in the solution. We performed real-time PCR to detect the 16S rRNA gene of *H. pylori*. In order to detect the East Asian *cagA* genotype, nested PCR was performed using genotype-specific primers. We performed two rounds of PCR. The first round was performed using a common forward primer (F1) and either of the two reverse primers (R1 or R2) (Fig. 1b, c). The PCR cycling conditions for the first round were as follows: 95 °C for 10 min, then 40 cycles at 94 °C for 15 s, 55 °C for 30 s and 68 °C for 30 s. The second round was performed using 1 µl of the PCR products obtained in the first round as the template. In the second round, primers specific to these two types were used in separate reactions. The cycling conditions of the second round of PCR were as follows: 94 °C for 2 min, then 30 cycles at 98 °C for 10 s and 63 °C for 30 s. The PCR products were electrophoresed on a 2 % agarose gel and visualized by ethidium bromide staining.

Data analysis. All results were analysed by χ^2 analysis. The level of statistical significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

Prevalence of *H. pylori*

In Japan, there has been a considerable increase in the incidence of gastric cancer in middle-aged individuals (Yoshida *et al.*, 2006); hence, for this study, middle-aged and older individuals who did not exhibit any subjective symptoms and had not received medical treatment in the previous 3 months were enrolled. The age distribution of the 186 participants enrolled in this study is shown in Table 2. Of the 186 participants, 75 (40.3 %) tested positive for the *H. pylori* antigen (Table 3). The incidence of *H. pylori* infection in each age group ranged from 33.3 to 51.2 %; no significant difference was observed among the age groups in this regard (Table 2).

The study revealed that a certain number of healthy asymptomatic Japanese individuals who were older than 39 years were infected with *H. pylori*; this was directly determined using the individuals' stool specimens. However, previous studies that employed the serological method reported higher incidences (70–80 %) of *H. pylori* infection among individuals who were ⩾40 years old (Asaka *et al.*, 1992; Fujisawa *et al.*, 1999; Yamagata *et al.*, 2000). It is likely that the difference between the present results and those reported by previous studies may be attributable to the different detection systems employed in these studies. The serological method may tend to yield a relatively higher infection rate than the antigen detection method using stool specimens because the serological method also detects past and cured infections. It has been shown that the sensitivity and specificity of the antigen detection method used in this study are comparable to those of the breath test, which is widely used as a standard method for the detection of *H. pylori* infection (Cardenas *et al.*, 2008).

Detection of the *H. pylori* 16S rRNA gene

Total bacterial genomic DNA was extracted from stool specimens that tested positive for the *H. pylori* antigen. In order to confirm the existence of *H. pylori* genomic DNA in the extracted DNA, real-time PCR was performed using

Table 1. Oligonucleotide primers and a probe used for PCR analysis

Gene	Primer/probe	Sequence	Reference
16S rRNA	Forward	5′-TGC GAA GTG GAG CCA ATC TT-3′	Yamazaki *et al.* (2005a)
	Reverse	5′-GGA ACG TAT TCA CCG CAA CA-3′	Yamazaki *et al.* (2005a)
	Probe	5′-(FAM) CCT CTC AGT TCG GAT TGT AGG CTG CAA C (TAMRA)-3′	Yamazaki *et al.* (2005a)
cagA	F1	5′-GGA ACC CTA GTC AGT AAT GGG TT-3′	Hirai *et al.* (2009)
	F2	5′-CCA ATA ACA ATA ATA ATG GAC TCA A-3′	This study
	R1	5′-GCT TTA GCT TCT GAT ACC GCT TGA-3′	Hirai *et al.* (2009)
	R2	5′-AAT TCT TGT TCC CTT GAA AGC CC-3′	Hirai *et al.* (2009)
	EA-F	5′-AAA GGA GTG GGC GGT TTC A-3′	Yamazaki *et al.* (2005a)
	EA-R	5′-CCT GCT TGA TTT GCC TCA TCA-3′	Yamazaki *et al.* (2005a)

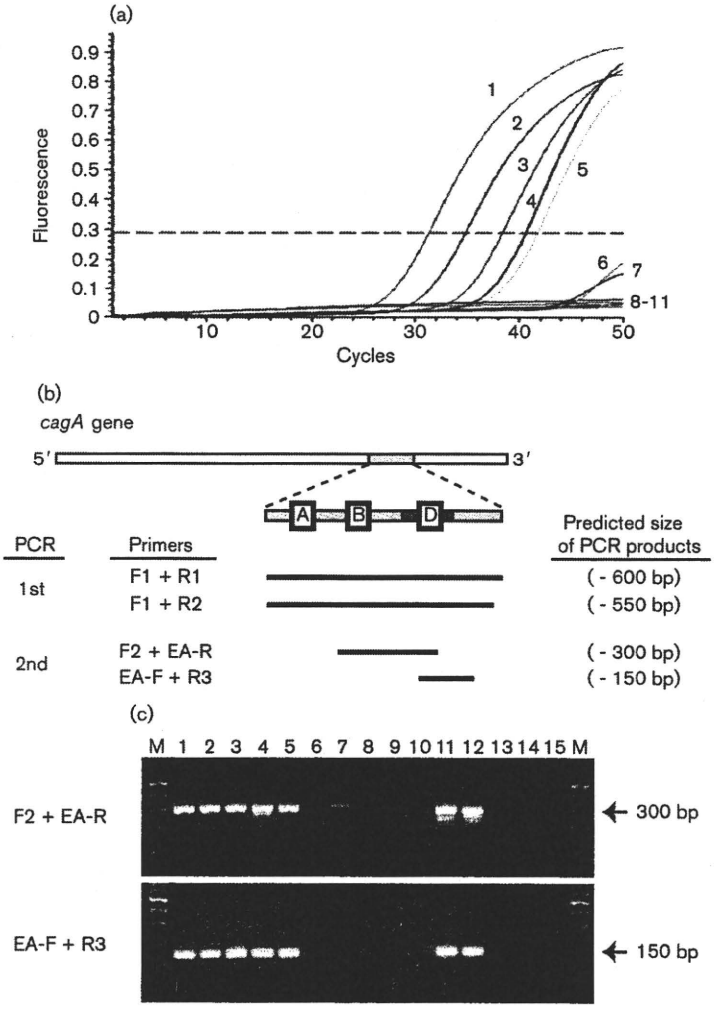

Fig. 1. Real-time PCR analysis of the 16S rRNA gene and genotyping of *cagA* encoding the virulence factor. (a) Representative amplification plots of real-time PCR targeting the 16S rRNA gene. Curves: 1–5, *H. pylori* DNA-positive specimens; 6–10, *H. pylori* DNA-negative specimens; 11, negative control (water); broken line, threshold cycle line. (b) Diagram showing the positions of the PCR products on the *cagA* gene. Alignment of the primer pairs used for genotyping and the sizes of PCR products are shown. (c) Representative detection of PCR products by agarose gel electrophoresis. Arrows indicate the expected sizes of the PCR products. Lanes: 1–5, East Asian *cagA* specimens; 6 and 7, Western *cagA* specimens; 8 and 9, unclassified *cagA* specimens; 10, a *cagA*-negative specimen; 11 and 12, DNA of East Asian *cagA*-positive *H. pylori* strains used as reference; 13 and 14, DNA of Western *cagA*-positive *H. pylori* strains used as reference; 15, negative control (water); M, molecular marker.

primers targeting the 16S rRNA gene (Yamazaki *et al.*, 2005a). The detection limit for the 16S rRNA gene was 10 copies μg^{-1}. As shown in Table 3, the 16S rRNA gene was detected in 65 of 75 (86.7 %) DNA samples obtained from stool samples that tested positive for the *H. pylori* antigen. This high detection rate indicated that the method used for DNA extraction was effective. In addition, *H. pylori* DNA was detected in fewer than 5 % of the DNA samples extracted from randomly selected stool specimens that tested negative for the *H. pylori* antigen. These findings indicate that the results of the present study revealed the prevalence of *H. pylori* infection in all the study participants.

Table 2. Prevalence of *H. pylori* infection and the ratio of the East Asian *cagA* genotype in the age groups

Age group	No. tested	No. antigen positive (%)	No. East Asian genotype positive (%)
40–45	55	20 (36.4)	8 (14.5)
46–50	42	16 (38.1)	11 (26.2)
51–55	42	16 (38.1)	7 (16.7)
56–60	41	21 (51.2)	13 (31.7)
>60	6	2 (33.3)	2 (33.3)

Table 3. Summary of *H. pylori* antigen, DNA and *cagA* detection

	No. tested	No. positive (%)
H. pylori antigen test	186	75 (40.3)
Real-time PCR 16S rRNA	75	65 (86.7)
Genotyping of *cagA* East Asian genotype	65	41 (63.1)

Detection of the East Asian *cagA* genotype

The East Asian *cagA* genotype was detected by performing nested PCR using the DNA samples extracted from the participants' stool specimens (Fig. 1b, c). First, using two pairs of gene-specific primers, we amplified a region at the 3′-end of the *cagA* gene that codes for multiple EPIYA phosphorylation motifs. Next, we confirmed the presence of the East Asian *cagA* genotype by performing two separate rounds of PCR using specific primer pairs (F2 + EA-R and FA-F + R3). As shown in Fig. 1(c), the specificity of the detection method was confirmed by using *H. pylori* strains with the East Asian *cagA* genotype as a reference. The East Asian *cagA* genotype was detected in 41 of 65 (63.1 %) genomic DNA samples of *H. pylori* (Table 3). The incidence of *cagA*-positive *H. pylori* ranged from 40.0 to 100.0 % across all age groups (Table 2). There was no significant difference between the age groups, except for the group comprising individuals aged ⩾60 years, because of the small sample number of participants in this age group.

The results of the present study indicated that 22.0 % of the healthy asymptomatic Japanese individuals participating in the study may be infected with the highly virulent *H. pylori* strain. A considerably higher number of healthy individuals were found to have infection with the highly virulent East Asian *cagA*-positive *H. pylori* in Japan than in Thailand [where 2.8 % (5/179) healthy asymptomatic individuals were positive for the highly virulent *H. pylori* infection; unpublished data]. The cause of the highly virulent *H. pylori* infection in a considerably high number of asymptomatic Japanese individuals is unknown. However, in a recent report, it has been suggested that (1) the geographical distribution of *H. pylori* strains harbouring a certain virulence factor genotype and (2) the incidence of cancer are responsible for the high incidence of *H. pylori* infection among asymptomatic Japanese individuals (Yamaoka *et al.*, 2008). The findings of Yamaoka *et al.* (2008) were based on genotype analysis of *H. pylori* strains that were clinically isolated from patients; however, their finding of a high incidence of gastric cancer in countries where the East Asian CagA is predominant is in agreement with the result obtained in our study.

A recent study showed that the eradication of *H. pylori* significantly suppressed the development of metachronous gastric cancer (Fukase *et al.*, 2008). The report does not directly suggest that the eradication of *H. pylori* infection in healthy asymptomatic individuals will suppress the onset of gastric cancer in the future, but it highlights the significance of *H. pylori* infection in gastric cancer development. Therefore, a silent infection with a highly virulent strain of *H. pylori*, such as one with the East Asian *cagA* genotype, in healthy individuals may be a critical public health issue in the prevention of gastric cancer.

To our knowledge, this is the first report on the prevalence of *H. pylori* infection among healthy asymptomatic Japanese individuals that describes results that were directly revealed by genetic analyses. We found a relatively high incidence of infection with the highly virulent *H. pylori* strain among asymptomatic adult Japanese individuals. Further investigations with a larger number of participants are required to determine precisely the significance of *H. pylori* with a virulence factor genotype in the development of gastric cancer.

ACKNOWLEDGEMENTS

This work was supported in part by the Program of Funding Research Centers for Emerging and Reemerging Infectious Diseases, MEXT, Japan.

REFERENCES

Asaka, M., Kimura, T., Kudo, M., Takeda, H., Mitani, S., Miyazaki, T., Miki, K. & Graham, D. Y. (1992). Relationship of *Helicobacter pylori* to serum pepsinogens in an asymptomatic Japanese population. *Gastroenterology* **102**, 760–766.

Azuma, T. (2004). *Helicobacter pylori* CagA protein variation associated with gastric cancer in Asia. *J Gastroenterol* **39**, 97–103.

Cardenas, V. M., Dominguez, D. C., Puentes, F. A., Aragaki, C. C., Goodman, K. J., Graham, D. Y. & Fukuda, Y. (2008). Evaluation of a novel stool native catalase antigen test for *Helicobacter pylori* infection in asymptomatic North American children. *J Pediatr Gastroenterol Nutr* **46**, 399–402.

Chattopadhyay, S., Datta, S., Chowdhury, A., Chowdhury, S., Mukhopadhyay, A. K., Rajendran, K., Bhattacharya, S. K., Berg, D. E. & Nair, G. B. (2002). Virulence genes in *Helicobacter pylori* strains from West Bengal residents with overt *H. pylori*-associated disease and healthy volunteers. *J Clin Microbiol* **40**, 2622–2625.

Covacci, A., Censini, S., Bugnoli, M., Petracca, R., Burroni, D., Macchia, G., Massone, A., Papini, E., Xiang, Z. & other authors (1993). Molecular characterization of the 128-kDa immunodominant antigen of *Helicobacter pylori* associated with cytotoxicity and duodenal ulcer. *Proc Natl Acad Sci U S A* **90**, 5791–5795.

Devi, S. M., Ahmed, I., Khan, A. A., Rahman, S. A., Alvi, A., Sechi, L. A. & Ahmed, N. (2006). Genomes of *Helicobacter pylori* from native Peruvians suggest admixture of ancestral and modern lineages and reveal a western type *cag*-pathogenicity island. *BMC Genomics* **7**, 191.

Fujisawa, T., Kumagai, T., Akamatsu, T., Kiyosawa, K. & Matsunaga, Y. (1999). Changes in seroepidemiological pattern of *Helicobacter pylori* and hepatitis A virus over the last 20 years in Japan. *Am J Gastroenterol* **94**, 2094–2099.

Fukase, K., Kato, M., Kikuchi, S., Inoue, K., Uemura, N., Okamoto, S., Terao, S., Amagai, K., Hayashi, S. & Asaka, M. (2008). Effect of eradication of *Helicobacter pylori* on incidence of metachronous gastric carcinoma after endoscopic resection of early gastric cancer: an open-label, randomised controlled trial. *Lancet* **372**, 392–397.

Higashi, H., Tsutsumi, R., Fujita, A., Yamazaki, S., Asaka, M., Azuma, T. & Hatakeyama, M. (2002a). Biological activity of the *Helicobacter pylori* virulence factor CagA is determined by variation in the tyrosine phosphorylation sites. *Proc Natl Acad Sci U S A* **99**, 14428–14433.

Higashi, H., Tsutsumi, R., Muto, S., Sugiyama, T., Azuma, T., Asaka, M. & Hatakeyama, M. (2002b). SHP-2 tyrosine phosphatase as an intracellular target of *Helicobacter pylori* CagA protein. *Science* **295**, 683–686.

Hirai, I., Sasaki, T., Fujimoto, S., Moriyama, T., Azuma, T. & Yamamoto, Y. (2009). A method for assessment of *Helicobacter pylori*

genotype using stool specimens. *FEMS Immunol Med Microbiol* **56**, 63–66.

Kanada, R., Uchida, T., Tsukamoto, Y., Nguyen, L. T., Hijiya, N., Matsuura, K., Kodama, M., Okimoto, T., Murakami, K. & other authors (2008). Genotyping of the *cagA* gene of *Helicobacter pylori* on immunohistochemistry with East Asian CagA-specific antibody. *Pathol Int* **58**, 218–225.

Molnar, B., Szoke, D., Ruzsovics, A. & Tulassay, Z. (2008). Significantly elevated *Helicobacter pylori* density and different genotype distribution in erosions as compared with normal gastric biopsy specimen detected by quantitative real-time PCR. *Eur J Gastroenterol Hepatol* **20**, 305–313.

Naito, M., Yamazaki, T., Tsutsumi, R., Higashi, H., Onoe, K., Yamazaki, S., Azuma, T. & Hatakeyama, M. (2006). Influence of EPIYA-repeat polymorphism on the phosphorylation-dependent biological activity of *Helicobacter pylori* CagA. *Gastroenterology* **130**, 1181–1190.

Suzuki, N., Wakasugi, M., Nakaya, S., Okada, K., Mochida, R., Sato, M., Kajiyama, H., Takahashi, R., Hirata, H. & other authors (2002). Production and application of new monoclonal antibodies specific for a fecal *Helicobacter pylori* antigen. *Clin Diagn Lab Immunol* **9**, 75–78.

Vilaichone, R. K., Mahachai, V., Tumwasorn, S., Wu, J. Y., Graham, D. Y. & Yamaoka, Y. (2004). Molecular epidemiology and outcome of *Helicobacter pylori* infection in Thailand: a cultural cross roads. *Helicobacter* **9**, 453–459.

Yamagata, H., Kiyohara, Y., Aoyagi, K., Kato, I., Iwamoto, H., Nakayama, K., Shimizu, H., Tanizaki, Y., Arima, H. & other authors (2000). Impact of *Helicobacter pylori* infection on gastric cancer incidence in a general Japanese population: the Hisayama study. *Arch Intern Med* **160**, 1962–1968.

Yamaoka, Y., Malaty, H. M., Osato, M. S. & Graham, D. Y. (2000a). Conservation of *Helicobacter pylori* genotypes in different ethnic groups in Houston, Texas. *J Infect Dis* **181**, 2083–2086.

Yamaoka, Y., Osato, M. S., Sepulveda, A. R., Gutierrez, O., Figura, N., Kim, J. G., Kodama, T., Kashima, K. & Graham, D. Y. (2000b). Molecular epidemiology of *Helicobacter pylori*: separation of *H. pylori* from East Asian and non-Asian countries. *Epidemiol Infect* **124**, 91–96.

Yamaoka, Y., Kato, M. & Asaka, M. (2008). Geographic differences in gastric cancer incidence can be explained by differences between *Helicobacter pylori* strains. *Intern Med* **47**, 1077–1083.

Yamazaki, S., Kato, S., Matsukura, N., Ohtani, M., Ito, Y., Suto, H., Yamazaki, Y., Yamakawa, A., Tokudome, S. & other authors (2005a). Identification of *Helicobacter pylori* and the *cagA* genotype in gastric biopsies using highly sensitive real-time PCR as a new diagnostic tool. *FEMS Immunol Med Microbiol* **44**, 261–268.

Yamazaki, S., Yamakawa, A., Okuda, T., Ohtani, M., Suto, H., Ito, Y., Yamazaki, Y., Keida, Y., Higashi, H. & other authors (2005b). Distinct diversity of *vacA*, *cagA*, and *cagE* genes of *Helicobacter pylori* associated with peptic ulcer in Japan. *J Clin Microbiol* **43**, 3906–3916.

Yoshida, S., Kozu, T., Gotoda, T. & Saito, D. (2006). Detection and treatment of early cancer in high-risk populations. *Best Pract Res Clin Gastroenterol* **20**, 745–765.

Bone 45 (2009) 949–955

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

The impact of diabetes mellitus on vitamin D metabolism in predialysis patients

Hirotaka Tanaka [a], Takayuki Hamano [a,*], Naohiko Fujii [b], Kodo Tomida [a], Isao Matsui [a], Satoshi Mikami [c], Yasuyuki Nagasawa [a], Takahito Ito [a], Toshiki Moriyama [a], Masaru Horio [a], Enyu Imai [a], Yoshitaka Isaka [a], Hiromi Rakugi [a]

[a] Department of Geriatric Medicine and Nephrology, Osaka University Graduate School of Medicine, B6, 2-2 Yamadaoka, Suita, Osaka, Japan
[b] Department of Nephrology, Hyogo Prefectural Hospital Nishinomiya, Hyogo, Japan
[c] Department of Internal Medicine, Higashi-Kori Hospital, Osaka, Japan

ARTICLE INFO

Article history:
Received 10 March 2009
Revised 2 June 2009
Accepted 16 July 2009
Available online 23 July 2009

Edited by: T. Matsumoto

Keywords:
25-hydroxyvitamin D
1-84 PTH
FGF-23
Calcitriol
Proteinuria
Diabetes mellitus

ABSTRACT

Although diabetes mellitus (DM) disturbs bone metabolism, little is known concerning its effects on laboratory abnormalities in chronic kidney disease-mineral and bone disorders (CKD-MBD). We extracted data for 602 patients from the Osaka Vitamin D Study in patients with CKD (OVIDS-CKD), an observational study enrolling predialysis outpatients. No enrolled patients received vitamin D, bisphosphonate, estrogen or raloxifene. We measured 1–84 PTH, 25-hydroxyvitamin D (25D), calcitriol, fibroblast growth factor-23 (FGF-23), calcium (Ca), and phosphate (P). Since there were 112 DM patients (group D), we extracted 112 age-, sex-, and eGFR-matched non-DM counterparts (group N). We compared biochemical markers between groups, and then performed multiple regression analyses for all 602 subjects to confirm the results obtained. Group D had significantly higher corrected Ca and P than group N throughout all stages of CKD. In group D, 25D decreased as renal function declined, while in group N it remained constant (interaction $P<0.05$). Despite higher P and poorer vitamin D status in DM, there were no differences in 1–84 PTH level between group D and group N stratified by stage of CKD, resulting in significantly lower calcitriol levels in group D in late CKD. Multiple regression analyses revealed that DM was significantly associated with low vitamin D status even with adjustment for urinary protein, and that this poorer vitamin D status in DM was responsible for lower calcitriol level associated with DM. Despite higher P, lower FGF-23 in early CKD (stages 1 + 2) and comparable level of FGF-23 in late stages of CKD (stages 3, 4, and 5) were observed in group D. We interpreted these results to indicate that inappropriate production of FGF-23 in DM might explain higher serum phosphate in DM. Multiple regression analysis with adjustment for covariates confirmed an independent relationship between DM and low FGF-23, implying the existence of dysfunction or decreased density of osteocytes in DM. Given the origin of these phosphaturic hormones, DM may thus have markedly deleterious effects on parathyroid and bone. Poorer vitamin D status and higher CaP product might be partly responsible for functional and structural changes of vasculature, respectively, in DM.

Introduction

Use of the new umbrella concept chronic kidney disease-mineral and bone disorders (CKD-MBD) was advocated by Kidney Disease Improving Global Outcomes (KDIGO) [1]. Three factors, ectopic calcification, renal osteodystrophy, and laboratory abnormalities, are included in this novel concept. It is known that in predialysis patients diabetes mellitus (DM) significantly worsens vascular calcification, one of the types of ectopic ossification reported to be associated with morbidity and mortality in hemodialysis patients [2–5]. Moreover, DM has a marked impact on renal osteodystrophy, including suppression of serum parathyroid hormone (PTH) in hemodialysis patients [6]. However, no studies have reported the differences in laboratory abnormalities between diabetic and non-diabetic predialysis patients.

It is known that DM disturbs bone metabolism in subjects without CKD [7,8]. One of the characteristics of bone metabolism in DM patients is low bone turnover. Although Type 2 DM has no deleterious effect on bone mineral density because of this low rate of bone turnover, risk of bone fracture is reported to be higher in diabetic patients [9–11]. Diabetic hemodialysis patients have also been reported to have an increased incidence of hip fracture [12]. Osteoblast dysfunction induced by oxidative stress might explain the disturbance of bone metabolism in DM, since it would lead to impairment of bone formation [13].

In this study, we focused on the effects of DM on serum calcium, phosphate, and vitamin D metabolism with adjustment for estimated glomerular filtration rate (eGFR). In investigating vitamin D regula-

* Corresponding author. Fax: +81 6 6879 3857.
E-mail address: hamatea@medone.med.osaka-u.ac.jp (T. Hamano).

8756-3282/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bone.2009.07.016

tion, we measured two phosphaturic hormones, PTH and fibroblast growth factor-23 (FGF-23). In this cross-sectional, observational study enrolling DM patients and sex-, age-, and eGFR-matched non-DM patients, we found that DM patients had higher serum corrected calcium and phosphate levels and poorer vitamin D status with comparable levels of 1–84 PTH, resulting in lower serum calcitriol levels in advanced CKD. We also found that DM was a significant negative determinant for serum FGF-23.

Methods

Study population

We extracted data for 602 patients from the Osaka Vitamin D Study in patients with CKD (OVIDS-CKD), a multicenter cross-sectional observational study enrolling 751 CKD outpatients in Japan. All patients gave written informed consent to participate in the study. The Ethics Committee at Osaka University Graduate School of Medicine approved this study (approval number 07142). Subjects were eligible for enrollment if they had never received steroids, bisphosphonates, native or active vitamin D, or raloxifene as determined by medical history. Since there is a report that statins do not affect mineral metabolism in CKD including calcium, phosphorus and iPTH [14], we included them into the analysis. Of the total of 602 subjects enrolled, 112 patients had DM (group D) and met the criteria of the American Diabetes Association. We selected one sex-, age-, and eGFR-matched control patient without diabetes for each enrolled diabetic patient (group N) to obtain a new cohort of 224 patients. We selected them by computerization, leading to least possibility of bias. In brief, each diabetic patient was assigned a 4-digit index number which includes the information of sex, eGFR category in 10 mL/min/1.73 m^2 band, and age category in 10 year band. And for each diabetic patient, one non-diabetic patient with the same 4-digit index number was selected from the entire non-diabetic population by using computer (a 1:1 match). If this could not be done, the algorithm then proceeded sequentially to the next highest digit match (a 3-, 2-digit) on index number to make "next-best" matches.

In order to exclude the selection bias, we further performed sensitivity analysis, in which we created another cohort of non-diabetic patients by another equation formula.

Measured variables

Blood and urine samples were obtained at enrollment. Blood samples were drawn from ambulatory patients after an overnight fast. After 30 minute incubation, blood samples were centrifuged for serum separation and the serum was stored frozen at −80 °C until analysis. Blood chemistry parameters (blood urea nitrogen, creatinine, albumin, calcium, and inorganic phosphate) were measured by standard automated techniques. Estimated GFR was calculated according to revised MDRD formula [15]. Full-length 1–84 PTH was measured by a third-generation assay (Whole PTH, Scantibodies, Santee, CA, USA). The biologically active form of FGF-23 (intact FGF-23) was measured by a sandwich enzyme-linked immunosorbent assay system (Kainos Laboratories, Inc., Tokyo, Japan). Levels of serum calcitriol and 25-hydroxyvitamin D were measured using a TFB 1,25-hydroxyvitamin D RIA kit (Immunodiagnostic Systems Ltd., Boldon, UK) and a ^{125}I RIA kit (DiaSorin Inc., Stillwater, MN, USA), respectively. Bone-specific alkaline phosphatase (bone ALP) and serum cross-linked N-telopeptide of type I collagen (NTX) were assayed by using the Osteolinks-Bone ALP high-sensitivity diagnostic enzyme immunoassay (EIA) kit (Sumitomo Pharmaceuticals, Co., Osaka, Japan) and an OSTEOMARK ELIZA kit (Mochida pharmaceutical Co., Tokyo, Japan), respectively. We measured the bone mineral density (BMD) of the second to fourth lumbar vertebrae (L2–4) in addition to the femoral neck with a dual-energy X-ray absorptiometer (Discovery A, Hologic Inc., Bedford, MA, USA) in the posterior–anterior projection. Serum calcium level was corrected for Alb using the formula corrected calcium = total calcium + (4.0 − albumin) * 0.8 if Alb < 4.0 mg/dL. Urinary protein was measured semiquantitatively with a dipstick test.

Statistical analyses

Demographic factors and laboratory data for groups D and N were compared using *t*-tests or Wilcoxon rank-sum tests for continuous variables as appropriate, and the Pearson chi-square test for categorical variables. When dependent or explanatory variables had no linearity, they were log transformed (e.g. 1–84 PTH, FGF-23).

First, between-group analyses of these laboratory parameters were performed using a multiple linear regression model with eGFR and group as explanatory variables. This model involves analysis of covariance (ANCOVA) only when there is no interaction between eGFR and group. We performed post-hoc analysis regarding the difference within each CKD stage only if significant interaction was observed.

Second, we confirmed between-group differences in laboratory parameters by stepwise forward multivariate linear regression analyses for the total of 602 patients enrolled. Factors adjusted in the analysis of FGF-23 were age, sex, body mass index, corrected Ca, P, eGFR, 1–84 PTH, 25D, and 1,25D. In the analysis of 25D, two models were used, with and without urinary protein, with adjustment for the season of collection of blood samples, age, sex, BMI, corrected Ca, P, eGFR, 1–84 PTH, and FGF-23. The prevalences of semiquantitative urinary protein (uPro) dipstick testing across CKD stages were compared by chi-square test separately in each group. If a difference was significant, we proceeded to Cochrane–Armitage trend analysis to examine the association between CKD stage and the prevalence of uPro ≥3+. To take into account the contributions of urinary protein [16] and season [17,18] to 25D level, we converted them to dummy variables in multivariable analyses for vitamin D status. Dummy variables were constructed with no urinary protein and winter as references, respectively. Herein, spring denotes March to May, summer June to August, autumn September to November, and winter December to February. In another analysis of calcitriol, we also prepared two models with or without 25D, to determine whether poorer calcitriol levels in diabetic patients were actually due to poorer

Table 1
Demographic factors and laboratory findings for all subjects and matched groups.

Variable	All subjects	Group D	Group N	*P* value between groups
n [female%]	602 [34.7%]	112 [25%]	112 [25%]	1
Age (years)	64 (54–71)	66 (57–73)	66 (57–73)	0.9
BMI (kg/m^2)	23.2 (21.2–25.5)	23.9 (22.0–26.7)	22.6 (20.9–25.1)	0.02
Cr (mg/dL)	1.4 (1.1–2.1)	1.9 (1.3–3.2)	1.8 (1.2–3.0)	0.6
Alb (g/dL)	4.0 (3.7–4.2)	3.9 (3.5–4.2)	4.0 (3.7–4.2)	0.08
eGFR (mL/min/1.73 m^3)	48.1 (30.3–65.9)	34.6 (19.0–55.1)	35.6 (20.5–58.7)	0.6
$uPro^N$ (−/±/+/2+/>3+)	126/83/116/93/61	13/17/26/22/32	19/15/24/11/8	0.007

n, numbers of patients; DM, diabetes mellitus; values are the mean ± SD or median (interquartile range) as appropriate.
Note. serum creatinine in mg/dL may be converted to μmol/L by multiplying by 88.4, albumin in g/dL to g/L by multiplying by 10, eGFR in mL/min/1.73 m^2 to mL/s/1.73 m^2 by multiplying by 0.01667.

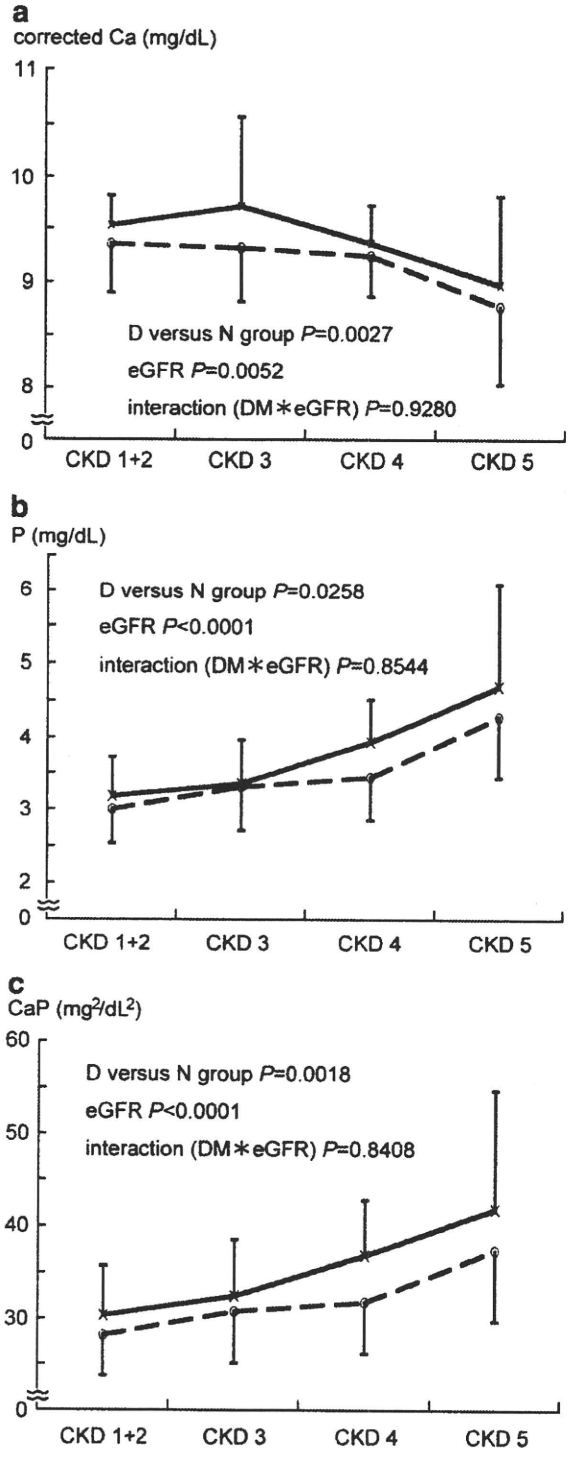

Fig. 1. Differences in laboratory findings between the two groups stratified by CKD stage. Differences between the two groups in corrected Ca (a), P (b), and CaP product (c) levels by CKD stage. The broken line represents group N and the solid line group D. Each bar represents the mean ± SD for group D or mean-SD for group N. Serum corrected Ca in mg/dL may be converted to mmol/L by multiplying by 0.2495, P in mg/dL to mmol/L by multiplying by 0.3229.

vitamin D status. In all analyses, 1–84 PTH and FGF-23 levels were log-transformed because their distributions were skewed. Statistical tests were two-sided, and P values of less than 0.05 were considered significant. All statistical analyses were performed using JMP software, version 7.0.2 (SAS Institute, NC, USA).

Results

Patient characteristics

A total of 602 subjects were analyzed, 34.7% ($n = 209$) of whom were female. Since there were 112 DM patients (group D), we extracted age-, sex-, and eGFR-matched non-DM counterparts (group N) to obtain a new cohort of 224 patients. The demographic factors and laboratory data in each group are shown in Table 1. Compared with group N, group D had higher body mass index (BMI) and more severe proteinuria, though the difference between the groups in serum albumin (Alb) was not statistically significant. Thirteen percent of non-diabetic patients (14 out of 112) received statins, whereas 38% of the diabetic patients (42 out of 112) received them. Only small portion of diabetic patients (8 out of 112) had thiazolidinedione prescribed, and 40% of the diabetic patients (46 out of 112) received insulin therapy. Regarding diuretics, thiazide and loop diuretics were

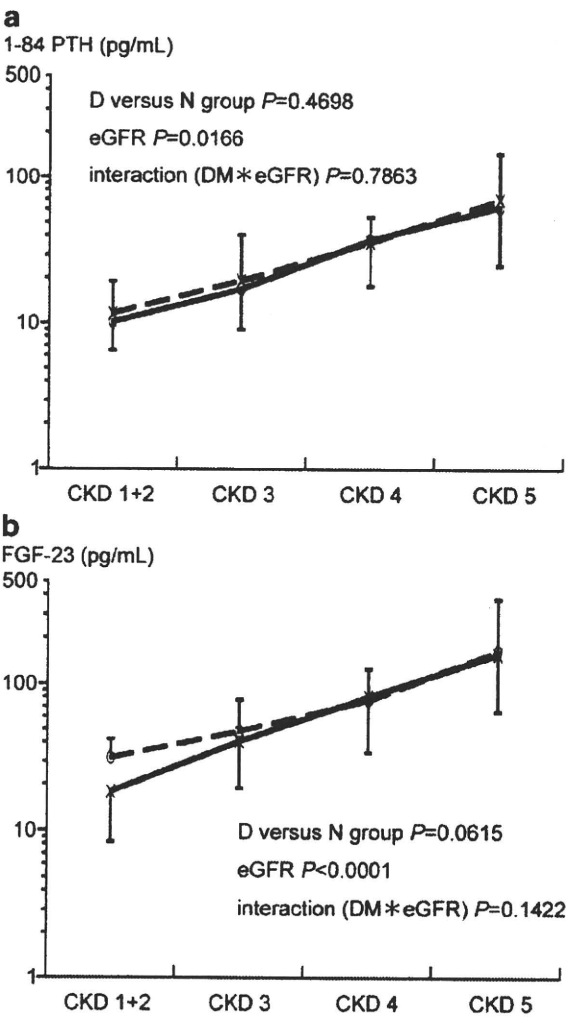

Fig. 2. Differences in phosphaturic hormones between the two groups by CKD stage. Differences between the two groups in 1–84 PTH (a) and FGF-23 (b) level by CKD stage. The broken line represents group N and the solid line group D. Each bar represents the mean ± SD for group N or mean-SD for group D. Serum 1–84 PTH levels in pg/mL and ng/L are equivalent.

Table 2
Multiple linear regression analyses for FGF-23.

Independent variable	β	SE	T	P
DM	−0.1242	0.0312	−3.98	<0.001
Sex (female)	−0.0637	0.0264	−2.42	0.02
eGFR	−0.0009	0.0015	−6.06	<0.001
Log (1–84 PTH)	0.1357	0.0363	3.74	<0.001
P	0.3071	0.0394	7.80	<0.001
25D	0.0127	0.0044	2.89	0.004
1,25D	−0.0092	0.0016	−5.60	<0.001
	Intercept = 2.82		$R^2 = 0.53$	

25D, 25-hydroxyvitamin D; 1,25D, 1,25-dihydroxyvitamin D; PTH, parathyroid hormone.

significantly more often used in group D (thiazide; 12% in group D, 4% in group N, $P<0.05$, loop; 47% in group D, 13% in group N, $P<0.0001$).

Differences in serum Ca and P between groups stratified by renal function

We compared biochemical laboratory findings in the two groups. Corrected calcium (Ca) was higher in group D ($P=0.0027$) than group N through all CKD stages, and decreased in both groups as renal function declined ($P=0.0052$). No significant interaction was detected (Fig. 1a). Serum phosphate (P) was also significantly higher in group D ($P=0.0258$), but increased in both groups as renal function declined (Fig. 1b); once again, interaction was not significant. Consequently, Ca P product, which was reported to be associated with the extent of vascular calcification in hemodialysis patients [19–21], was significantly higher in group D ($P=0.0018$), and increased in both groups as renal function declined. Again, no significant interaction was observed (Fig. 1c).

Two phosphaturic hormones

In the same fashion, we examined the difference between groups in two phosphaturic hormones, 1–84 PTH and full length FGF-23. Levels of both hormones increased as renal function declined, with no interaction observed. No significant differences were found in levels of either phosphaturic hormone between the groups (Figs. 2a, b), despite higher serum phosphate level in group D. Moreover, there was a trend toward lower FGF-23 in group D in early CKD (stages 1 + 2), though this difference decreased in late CKD. Thus, since group D had significantly higher phosphate than group N, our finding that group D had a borderline significantly lower FGF-23 overall ($P=0.06$) was paradoxical. In this context, we examined the relationship between DM and low FGF-23 by multiple regression analysis of FGF-23 in the group of all enrolled patients, with adjustment for serum phosphate (Table 2), and found that DM was significantly and

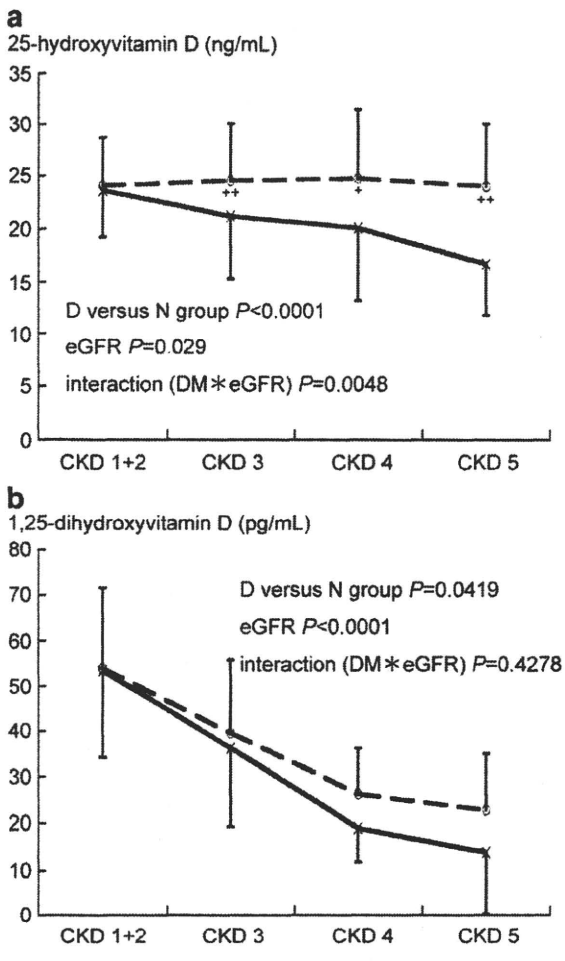

Fig. 3. Differences in vitamin D status and serum calcitriol level between the two groups by CKD stage. Differences between the two groups in 25-hydroxyvitamin D (a) and 1,25-dihydroxyvitamin D (b) level by CKD stage. The broken line represents group N and the solid line group D. Each bar represents the mean + SD for group N or mean-SD for group D. +, $P<0.05$; ++, $P<0.01$: results of post-hoc analysis for comparison between groups within each CKD stage. Serum 25-hydroxyvitamin D in ng/mL may be converted to nmol/L by multiplying by 2.496; serum 1,25-dihydroxyvitamin D in pg/mL to pmol/L by multiplying by 2.6.

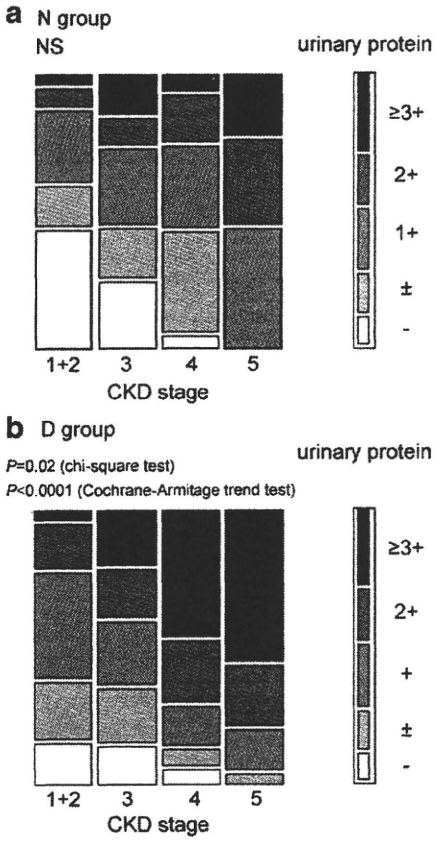

Fig. 4. The prevalence of proteinuria category as determined by dipstick test in each CKD stage by DM status. Urinary protein was measured semiquantitatively by dipstick test. (b) The differences in amount of proteinuria across CKD stages were significant in group D ($P=0.02$ by chi-square test) but not in group N (a). There was also a significant trend toward increase in percentage of patients with urinary protein ≥ 3+ with progression of CKD stage ($P<0.0001$ by Cochrane-Armitage trend test). NS means not significant.

Table 3
Multiple linear regression analyses for 25-hydroxyvitam D.

Independent variable	Model 1		Model 2	
	β	P	β	P
DM	−1.6547	<0.001	−1.1502	<0.001
Sex (female)	−0.8790	<0.001	−1.0879	<0.001
Log (1–84 PTH)	−1.1829	<0.001	−0.9413	0.001
Season (spring and autumn)	0.0843	0.8	0.1139	0.7
Season (summer)	1.2824	<0.001	1.3004	<0.001
uPro (±)	-	-	−0.3564	0.3
uPro (1+)	-	-	−0.4953	0.1
uPro (2+)	-	-	−0.8654	0.01
uPro (≥3+)	-	-	−2.3371	<0.001
	$R^{2\#}=0.11$		$R^{2\#}=0.15$	

#, corrected R^2 for degree of freedom.
uPro, urinary protein; PTH, parathyroid hormone.
Reference of the seasons was set at winter, reference of uPro was no urinary protein, i.e. (−) by dipstick tests.
Model 1 is a model without uPro, Model 2 with uPro.

independently associated with low FGF-23, with adjustment for numerous confounders such as eGFR and serum phosphate.

Impact of DM and renal function on vitamin D status and calcitriol

Group D had poorer vitamin D status (Fig. 3a) with comparable levels of 1–84 PTH (Fig. 2b), leading to lower calcitriol levels in comparison with group N (Fig. 3b). Marked difference between the groups was observed in the association between CKD stage and serum 25-hydroxyvitamin D (25D) level. In group D, 25D decreased as renal function declined, while in group N it was constant through all CKD stages (Fig. 3a, interaction $P=0.048$). The percentage of patients with proteinuria ≥ +3 increased as renal function declined in group D ($P<0.0001$ by Cochrane–Armitage trend analysis), but was constant in group N (Fig. 4). It is known that in nephrotic syndrome 25D is lost in urine with vitamin D binding protein [22]. Therefore, attributing the difference in vitamin D status between the groups to their difference in degree of proteinuria, we performed multivariate linear regression analyses for 25D with adjustment for proteinuria. Model 2 including proteinuria as an explanatory variable had a higher corrected R^2 for degrees of freedom than model 1, which did not include proteinuria. In both models, however, DM remained an independent factor for poor vitamin D status (Table 3).

In order to confirm that lower serum calcitriol level in DM was due to poorer vitamin D status, we constructed two multivariate models for serum calcitriol with or without 25D, the substrate of 1,25-hydroxyvitamin D (1,25D). Inclusion of 25D (Model 2) eliminated the significant effect of DM on 1,25D found in Model 1 (Table 4).

Impact of DM on bone markers and bone mineral density

We could not observe any difference in serum BSAP or NTX level between the two groups (data not shown). Neither did we observe any difference in bone mineral density Z-score (at femoral neck or lumbar spine) (data not shown), although body mass index was significantly higher in diabetic patients.

The percentage of prior cardiovascular disease in both groups

Twenty-three percent of diabetic patients (26 out of 112) had prior cardiovascular disease (CVD) including myocardial infarction, angina pectoris, myocardial ischemia, whereas only 5% of non-diabetic patients (6 out of 112) had prior CVD.

Sensitivity analysis

As a sensitivity analysis, we made another matched non-DM group consisted of 112 patients. The same results were obtained regarding the differences in laboratory data between groups (data not shown).

Discussion

We investigated the effects of DM on laboratory parameters related to CKD-MBD in this cross-sectional study with enrollment of a sex-, age-, and eGFR-matched CKD cohort. The subjects of our study, who were mainly ambulatory patients who had never been treated for CKD-MBD, had never received vitamin D, estrogen, raloxifene, or bisphosphonate.. The following findings were obtained: (1) Compared with non-diabetic subjects, diabetic patients had higher serum corrected Ca and P levels and comparable levels of 1–84 PTH despite poor vitamin D status and higher serum P. (2) Multiple regression analysis adjusted for serum P and eGFR revealed a relationship between DM and low FGF-23. (3) Whereas serum 25D level was constant across CKD stages in non-DM patients, it deceased in patients with DM as CKD stage progressed. The relationship between poor vitamin D status and DM remained robust even after adjustment for degree of proteinuria. (4) Serum calcitriol was also significantly decreased in DM patients, especially in advanced CKD. Multiple

Table 4
Multiple linear regression analyses for 1,25-dihydroxyvitamin D.

Independent variable	Model 1		Model 2	
	β	P	β	P
DM	−2.4657	0.007	−1.0258	0.2
eGFR	0.3864	<0.001	0.3941	<0.001
Log (FGF-23)	−6.7567	<0.001	−7.1915	<0.001
Log (1–84 PTH)	2.3939	0.02	3.7110	<0.001
25D	-	-	0.8793	<0.001
	$R^{2\#}=0.40$		$R^{2\#}=0.47$	

#, corrected R^2 for degree of freedom.
25D, 25-hydroxyvitamin D; FGF-23, fibroblast growth factor-23; PTH, parathyroid hormone.
Model 1 is a model without 25D, Model 2 with 25D.

regression analysis implied that poor vitamin D status was a reason for the low serum calcitriol levels in DM.

Higher corrected serum Ca in DM was also reported in elderly patients above age 70 in nursing homes [23]. Higher CaP product in DM might be one of the reasons for the higher coronary artery calcification score reported in predialysis DM patients [5]. Although it is unclear how DM contributes to higher serum Ca despite lower vitamin D status. More frequent prescription of thiazide diuretics in group D might play a role in it. However, loop diuretics associated with negative calcium balance was also used more often in group D. Another possibility might be low bone turnover in DM. Inaba et al. reported in an *in vitro* study that exposure to high glucose impaired the response of human osteoblast-like cells to PTH [24] and 1,25D [25]. They also found that poor glycemic control impaired the response of bone formation markers to exogenous calcitriol in patients with type 2 DM [26]. Although BSAP and NTX level was comparable between the groups, we cannot say safely that low bone turnover was not present in group D, since we lack data on serum osteocalcin, which is reported to be negatively correlated with by $HbA1_C$ in diabetic patients [23]. Therefore potential low bone turnover in DM may lead to low buffering capacity of bone, resulting in higher serum Ca and P. Since FGF-23, one of the phosphaturic hormones, is produced mainly in osteocytes [27] under conditions of high serum P level [28,29]. Considering a report that body weight is positively associated with serum FGF-23 [30], group D patients with higher body weight should have had higher serum FGF-23, however, we found a independent link between DM and low FGF-23. Given that the plasma half-life of this hormone is in the range of 46–58 min [31], low serum FGF-23 can be attributed to its reduced production. Therefore, the finding of lower FGF-23 level in DM in early stages of CKD despite higher serum P implies that the function of osteocytes is disturbed or osteocyte density is reduced in DM. This result can be otherwise interpreted as indicating that impaired osteocyte function in DM resulted in relatively low FGF-23 levels, leading to higher serum P. In fact, osteocyte density was reported to be reduced in a rat model of acute DM [32], the mechanism of which may involve oxidative stress, as reported in osteoblasts [33]. It seems likely that uremia overcomes the effects of DM on FGF-23, resulting in comparable levels of FGF-23 in subjects with and without DM in late stages of CKD.

In our analysis, we revealed the link between lower FGF-23 and DM, which predisposes patients to high morbidity and mortality. Our results are not inconsistent with previous literature arguing that high serum cFGF-23 level at the initiation of hemodialysis therapy was reported to be associated with higher mortality risk [34]. They adjusted for DM in this brilliant manuscript, meaning that higher serum cFGF-23 was associated with higher mortality risk in DM and also in non-DM (regardless of diabetic state). They did not insist that higher mortality in DM was due to higher FGF-23 level in them. In fact, they did not show a result such that inclusion of FGF-23 into the model attenuated or disappeared the deleterious effect of DM on mortality.

A large difference in the dietary recipe between the group D and group N might explain the difference in serum P. In early CKD stages, no protein restriction was performed in diabetic patients, leading to higher protein intake than in non-diabetic CKD patients. Because of lower serum level of FGF-23 in CKD stage 1 + 2, urinary excretion of phosphate might not compensate for this higher phosphate intake, resulting in higher serum phosphate in this population.

Even though diabetic patients had higher serum P and lower 25D and 1,25D levels, they had levels of 1–84 PTH comparable to those in non-diabetic patients, suggesting that parathyroid function is also impaired in predialysis DM patients. These results accord with the finding of *in vitro* analysis that increasing medium concentration of glucose caused suppression of PTH secretion by cultured bovine parathyroid cells [35]. These findings together suggest that the function of parathyroid cells and bone cells may be impaired in DM.

The progression of proteinuria with decrease in eGFR in DM suggested that massive proteinuria is one of the reasons for poor vitamin D status in advanced CKD in patients with DM. In fact, inclusion of amount of proteinuria in the model increased the corrected R^2 for degrees of freedom. However, the relationship between DM and poor vitamin D status remained robust even after adjustment for proteinuria. The relationship between low vitamin D status and low insulin sensitivity reported for both the general population [36] and CKD patients [37] is consistent with our findings. As confirmed by multiple regression analysis, the low calcitriol level in advanced CKD in patients with DM was due to low 25D level. A clinical study reported that low 25D level at the initiation of hemodialysis is associated with poor prognosis [38], and another observational study by London et al. reported that nutritional vitamin D deficiency and low 1,25D could be associated with arteriosclerosis and endothelial dysfunction [39]. Therefore, the poorer vitamin D status and lower calcitriol level found in DM might contribute to the increased likelihood of cardiovascular disease in diabetic patients. In fact, the prevalence of the patients with prior CVD was as high as 23% in group D, whereas only 5% in group N.

One of the limitations of our study is that it was cross-sectional. We lack findings regarding the effects of control of blood glucose on the parameters we measured, and are thus unable to determine whether the DM-specific findings for laboratory parameters we detected are due to high insulin resistance, low serum level of insulin like growth factor-1, oxidative stress in DM, or high glucose level itself. Since an experimental study revealed that intensive control of blood glucose by insulin therapy resulted in improvement of bone turnover in a mouse model of type 1 DM [13], a clinical study in CKD is clearly needed to determine whether lowering of blood glucose by any agent will affect bone turnover or biochemical parameters associated with it.

Our examination of predialysis patients revealed some impacts of DM on laboratory abnormalities, one of the characteristics of CKD-MBD, including higher CaP product through all stages of CKD, poorer vitamin D status, lower serum calcitriol level in advanced CKD, and lower FGF-23 level. Poor vitamin D status and low calcitriol level in DM could bring about functional changes in the vasculature mediated by endothelial dysfunction, and high CaP product in DM could bring about structural changes in the vasculature, i.e. vascular calcification, another aspect of CKD-MBD. The finding of comparable levels of two phosphaturic hormones in subjects with and without DM despite higher phosphate and poor vitamin D status in those with DM implies impairment of bone and parathyroid function in DM.

References

[1] Moe S, Drueke T, Cunningham J, Goodman W, Martin K, Olgaard K, et al. Definition, evaluation, and classification of renal osteodystrophy: a position statement from Kidney Disease: Improving Global Outcomes (KDIGO). Kidney Int 2006;69:1945–53.

[2] Matsuoka M, Iseki K, Tamashiro M, Fujimoto N, Higa N, Touma T, et al. Impact of high coronary artery calcification score (CACS) on survival in patients on chronic hemodialysis. Clin Exp Nephrol 2004;8:54–8.

[3] Okuno S, Ishimura E, Kitatani K, Fujino Y, Kohno K, Maeno Y, et al. Presence of abdominal aortic calcification is significantly associated with all-cause and cardiovascular mortality in maintenance hemodialysis patients. Am J Kidney Dis 2007;49:417–25.

[4] Block GA, Raggi P, Bellasi A, Kooienga L, Spiegel DM. Mortality effect of coronary calcification and phosphate binder choice in incident hemodialysis patients. Kidney Int 2007;71:438–41.

[5] Kramer H, Toto R, Peshock R, Cooper R, Victor R. Association between chronic kidney disease and coronary artery calcification: the Dallas Heart Study. J Am Soc Nephrol 2005;16:507–13.

[6] Inaba M, Nagasue K, Okuno S, Ueda M, Kumeda Y, Imanishi Y, et al. Impaired secretion of parathyroid hormone, but not refractoriness of osteoblast, is a major mechanism of low bone turnover in hemodialyzed patients with diabetes mellitus. Am J Kidney Dis 2002;39:1261–9.

[7] Rosato MT, Schneider SH, Shapses SA. Bone turnover and insulin-like growth factor I levels increase after improved glycemic control in noninsulin-dependent diabetes mellitus. Calcif Tissue Int 1998;63:107–11.

[8] Achemlal L, Tellal S, Rkiouak F, Nouijai A, Bezza A, Derouiche el M, et al. Bone metabolism in male patients with type 2 diabetes. Clin Rheumatol 2005;24:493–6.
[9] Ivers RQ, Cumming RG, Mitchell P, Peduto AJ. Diabetes and risk of fracture: the Blue Mountains Eye Study. Diabetis Care 2001;24:1198–203.
[10] Vestergaard P. Discrepancies in bone mineral density and fracture risk in patients with type 1 and type 2 diabetes—a meta-analysis. Osteoporos Int 2007;18:427–44.
[11] Yamamoto M, Yamaguchi T, Yamauchi M, Kaji H, Sugimoto T. Bone mineral density is not sensitive enough to assess the risk of vertebral fractures in type 2 diabetic women. Calcif Tissue Int 2007;80:353–8.
[12] Danese MD, Kim J, Doan QV, Dylan M, Griffiths R, Chertow GM. PTH and the risks for hip, vertebral, and pelvic fractures among patients on dialysis. Am J Kidney Dis 2006;47:149–56.
[13] Hamada Y, Kitazawa S, Kitazawa R, Fujii H, Kasuga M, Fukagawa M. Histomorphometric analysis of diabetic osteopenia in streptozotocin-induced diabetic mice: a possible role of oxidative stress. Bone 2007;40:1408–14.
[14] Ashman N, Banerjee A, Yaqoob MM. Statins do not affect mineral metabolism in chronic kidney disease: a retrospective analysis. Nephron Clin Pract 2009;111(4):c236–9.
[15] Levey AS, Coresh J, Greene T, Stevens LA, Zhang YL, Hendriksen S, et al. Using standardized serum creatinine values in the modification of diet in renal disease study equation for estimating glomerular filtration rate. Ann Intern Med 2006;145:247–54.
[16] Mittal SK, Dash SC, Tiwari SC, Agarwal SK, Saxena S, Fishbane S. Bone histology in patients with nephrotic syndrome and normal renal function. Kidney Int 1999;55:1912–9.
[17] Salamone LM, Dallal GE, Zantos D, Makrauer F, Dawson-Hughes B. Contributions of vitamin D intake and seasonal sunlight exposure to plasma 25-hydroxyvitamin D concentration in elderly women. Am J Clin Nutr 1994;59:80–6.
[18] Mowe M, Bohmer T, Haug E. Serum calcidiol and calcitriol concentrations in elderly people: variations with age, sex, season and disease. Clin Nutr 1996;15:201–6.
[19] Goodman WG, Goldin J, Kuizon BD, Yoon C, Gales B, Sider D, et al. Coronary-artery calcification in young adults with end-stage renal disease who are undergoing dialysis. N Engl J Med 2000;342:1478–83.
[20] Cozzolino M, Brancaccio D, Gallieni M, Slatopolsky E. Pathogenesis of vascular calcification in chronic kidney disease. Kidney Int 2005;68:429–36.
[21] Cozzolino M, Dusso AS, Slatopolsky E. Role of calcium-phosphate product and bone-associated proteins on vascular calcification in renal failure. J Am Soc Nephrol 2001;12:2511–6.
[22] Hosogane N, Shinki T, Kasuga H, Taketomi S, Toyama Y, Suda T. Mechanisms for the reduction of 24,25-dihydroxyvitamin D3 levels and bone mass in 24-hydroxylase transgenic rats. Faseb J 2003;17:737–9.
[23] Dobnig H, Piswanger-Solkner JC, Roth M, Obermayer-Pietsch B, Tiran A, Strele A, et al. Type 2 diabetes mellitus in nursing home patients: effects on bone turnover, bone mass, and fracture risk. J Clin Endocrinol Metab 2006;91:3355–63.
[24] Yoshida O, Inaba M, Terada M, Shioi A, Nishizawa Y, Otani S, et al. Impaired response of human osteosarcoma (MG-63) cells to human parathyroid hormone induced by sustained exposure to high glucose. Miner Electrolyte Metab 1995;21:201–4.
[25] Inaba M, Terada M, Koyama H, Yoshida O, Ishimura E, Kawagishi T, et al. Influence of high glucose on 1,25-dihydroxyvitamin D3-induced effect on human osteoblast-like MG-63 cells. J Bone Miner Res 1995;10:1050–6.
[26] Inaba M, Nishizawa Y, Mita K, Kumeda Y, Emoto M, Kawagishi T, et al. Poor glycemic control impairs the response of biochemical parameters of bone formation and resorption to exogenous 1,25-dihydroxyvitamin D3 in patients with type 2 diabetes. Osteoporos Int 1999;9:525–31.
[27] Yoshiko Y, Wang H, Minamizaki T, Ijuin C, Yamamoto R, Suemune S, et al. Mineralized tissue cells are a principal source of FGF23. Bone 2007;40:1565–73.
[28] Shigematsu T, Kazama JJ, Yamashita T, Fukumoto S, Hosoya T, Gejyo F, et al. Possible involvement of circulating fibroblast growth factor 23 in the development of secondary hyperparathyroidism associated with renal insufficiency. Am J Kidney Dis 2004;44:250–6.
[29] Gutierrez O, Isakova T, Rhee E, Shah A, Holmes J, Collerone G, et al. Fibroblast growth factor-23 mitigates hyperphosphatemia but accentuates calcitriol deficiency in chronic kidney disease. J Am Soc Nephrol 2005;16:2205–15.
[30] Marsell R, Mirza MA, Mallmin H, Karlsson M, Mellström D, Orwoll E, et al. Relation between fibroblast growth factor-23, body weight and bone mineral density in elderly men. Osteoporos Int 2008 Oct 31.
[31] Khosravi A, Cutler CM, Kelly MH, Chang R, Royal RE, Sherry RM, et al. Determination of the elimination half-life of fibroblast growth factor-23. J Clin Endocrinol Metab 2007;92:2374–7.
[32] Villarino ME, Sanchez LM, Bozal CB, Ubios AM. Influence of short-term diabetes on osteocytic lacunae of alveolar bone. A histomorphometric study. Acta Odontol Latinoam 2006;19:23–8.
[33] Bai XC, Lu D, Bai J, Zheng H, Ke ZY, Li XM, et al. Oxidative stress inhibits osteoblastic differentiation of bone cells by ERK and NF-kappaB. Biochem Biophys Res Commun 2004;314:197–207.
[34] Gutiérrez OM, Mannstadt M, Isakova T, Rauh-Hain JA, Tamez H, Shah A, et al. Fibroblast growth factor 23 and mortality among patients undergoing hemodialysis. N Engl J Med 2008 Aug 7;359(6):584–92.
[35] Sugimoto T, Ritter C, Morrissey J, Hayes C, Slatopolsky E. Effects of high concentrations of glucose on PTH secretion in parathyroid cells. Kidney Int 1990;37:1522–7.
[36] Chiu KC, Chu A, Go VL, Saad MF. Hypovitaminosis D is associated with insulin resistance and beta cell dysfunction. Am J Clin Nutr 2004;79:820–5.
[37] Chonchol M, Scragg R. 25-Hydroxyvitamin D, insulin resistance, and kidney function in the Third National Health and Nutrition Examination Survey. Kidney Int 2007;71:134–9.
[38] Wolf M, Shah A, Gutierrez O, Ankers E, Monroy M, Tamez H, et al. Vitamin D levels and early mortality among incident hemodialysis patients. Kidney Int 2007;72:1004–13.
[39] London GM, Guerin AP, Verbeke FH, Pannier B, Boutouyrie P, Marchais SJ, et al. Mineral metabolism and arterial functions in end-stage renal disease: potential role of 25-hydroxyvitamin D deficiency. J Am Soc Nephrol 2007;18:613–20.

ELSEVIER

Clinicopathological Study of Expression of Lymphatic Vessels in Renal Allograft Biopsy After Treatment for Acute Rejection

K. Oka, Y. Namba, N. Ichimaru, T. Moriyama, M. Kyo, Y. Kokado, E. Imai, and S. Takahara

ABSTRACT

Background. Lymph vessel expression is related to inflammatory cell infiltration, around renal tubules in acute rejection episodes (ARE) of transplanted kidneys. However, there is little information on the lymph vessels after treatment of an ARE, particularly in relation to renal function and histological findings.

Patients and Methods. We investigated 13 cases of ARE diagnosed by kidney transplant biopsy performed from 1997 to 2005 within 3 years of transplantation. Treatment of the ARE lead to an improved serum creatinine level in all cases. There was neither an ABO-incompatible nor an acute humoral rejection case. Lymphatic vessels in re-biopsies were examined using immunohistochemical staining with D2-40 antibody that detected lymphatic endothelium. Re-biopsy cases in which the baseline creatinine had increased by more than 20% despite treatment were considered the severe group; the others, as the stable group. The relation between lymphatic vessel density (LVD) and renal function was examined using Banff scores.

Results. LVD was significantly higher in the severe than the stable group. The expression of lymph vessels versus the Banff score showed a direct relation: greater Banff scores showed higher expressions of lymph vessels.

Conclusions. The expression of lymph vessels in renal allograft specimens after treatment of an ARE was related to deterioration of renal function and inflammatory cell invasion. We plan a further examination of the relationship between the expression of lymph vessels and long-term prognosis.

SEVERAL NEW, RELIABLE antibodies have been produced to show lymphatic vessels. In the human kidney, lymph vessels are present around arterial branches at the level of the interlobular arteries, but not in peritubular spaces or around glomeruli.[1] It has been reported that lymphatic neoangiogenesis is associated with cellular infiltration. In renal allograft cases of acute rejection episodes (ARE), lymphatic neoangiogenesis is located in peritubular spaces and around glomeruli.[1] Lymphatic neoangiogenesis appears soon after transplantation.[2] It has been speculated that lymphatic neoangiogenesis contributes to cell drainage maintaining alloimmune responses to the graft.[1] In this study, we examined the clinicopathological significance of lymph vessel expression in renal allograft tissue biopsies after treatment of an ARE.

METHODS

Patients and Tissue Samples

We have performed 671 renal allograft biopsies from 1997 to 2005, including 39 in patients diagnosed with ARE greater than grade 1. Furthermore, among 39 patients who had ARE treated fewer than 3 years prior, 13 subjects underwent protocol biopsies. (Table 1).

Their mean serum creatinine (s-Cr) at ARE was 2.1 ± 0.6 mg/dL. Following ARE, treatment s-Cr improved in all cases and no case showed ARE upon a re-biopsy. Cases in which s-Cr increased more than 20% after treatment at re-biopsy were considered the severe group; whereas, other cases were the stable group. There was neither an ABO-incompatible nor an acute humoral rejection case. The study protocol was approved by our ethics committee. Written informed consent was obtained from each patient who underwent a biopsy. No patient suffered from diabetes mellitus.

From the Osaka Kaisei Hospital, Osaka, Japan.

Address reprint requests to Kazumasa Oka, PhD, MD, Osaka Kaisei Hospital, 1-6-10 Miyahara, Yodogawaku, Osaka 532-0003, Japan. E-mail: okaokamura@cocoa.och.ne.jp

0041-1345/09/$–see front matter
doi:10.1016/j.transproceed.2009.09.067

Table 1. Clinical Finding Profiles of Patients

Sex Male 7, female 6
Grade of AR AR-IA, 9; AR-IB, 1; AR-IIA, 3
Days after transplantation to AR onset 107 ± 128
Period from diagnosis of AR to re-biopsy (y) 1.9 ± 1.2

Abbreviation: AR, acute rejection.

Immunohistochemistry

Lymphatic vessels were detected in paraffin sections using an antibody against lymphatic endothelium—the mouse monoclonal anti-D2-40 immunoglobulin G1 (#17730-23, Signet Laboratories Inc.). This novel monoclonal antibody recognizes a Mr. 40,000 O-linked sialoglycoprotein that reacts with a fixation-resistant epitope in lymphatic endothelium. D2-40 has been described as a reliable marker for lymphatic endothelium in paraffin-embedded tissue. It binds to human podoplanin.

Immunohistochemical staining for D2-40 was performed as described previously: histological specimens fixed in 10% buffered-formalin were routinely processed for paraffin embedding, and serial 2-μm sections that were deparaffinized, were heated in an autoclave in 0.01 M citric acid buffer (pH 6.4; 121°C/15 min), incubated with 0.3% hydrogen peroxide in methanol for 15 minutes at room temperature to inhibit endogenous peroxidase activity, and washed in 0.05 M phosphate buffer (pH 7.6) 3 times for 3 minutes at room temperature before treatment with PBA for 5 minutes to block nonspecific staining. The sections were incubated with the mouse antihuman D2-40 monoclonal antibody (1:500) overnight at 4°C. After washing in 0.05 M phosphate buffer (pH 7.6) 3 times at room temperature for 3 minutes, they were processed using an avidin-biotinylated peroxidase complex method (Vectastain ABC kit, Vector Laboratories, Inc., Burlingame, California, United States) with diaminobenzidine as the chromogen and counter-stained with hematoxylin.

Quantitative Analysis of D2-40 Staining

The expression level of lymphatic vessels was evaluated based on the method previous reported by Yamamoto et al.[2] **The lymphatic vessel density (LVD) in biopsies was quantified as the number of D2-40 positive vascular profiles per unit area of the cortex using image analysis (MacScope). The analyses were performed by two of the authors (K.O., Y.N.) without prior knowledge of patients' characteristics.**

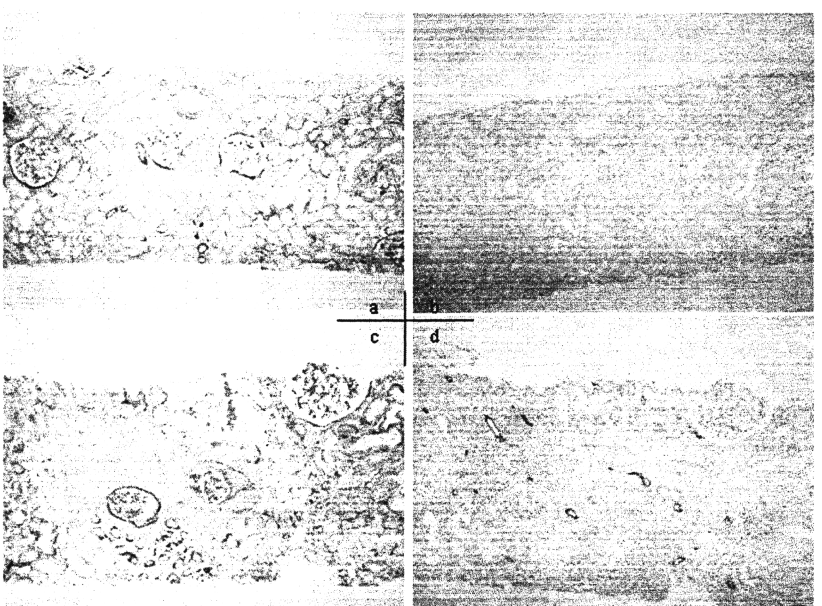

Fig 1. Stable group [**(A)** PAS staining × 100, **(B)** immunohistochemistry staining of D2-40]. Severe group [**(C)** PAS staining × 100, **(D)** immunohistochemistry staining of D2-40].

Histological Evaluation

We used the Banff97 system to evaluate renal allograft biopsies and to explore a relation to LVD and renal function.

Statistical Analysis

Results were expressed as mean values ± SEM. The Mann-Whitney *U* test was used to compare unpaired means of the 2-groups with differences evaluated using Stat View software (Abacus Concepts Inc., Berkeley, California, United States). *P* values <.05 were considered to be significant.

RESULTS

Among 13 patients, the stable group included 10 patients, and the severe group, 3 patients. The expression of lymph vessels in the stable group was mainly observed around blood vessels. In the severe group, it was recognized around cortical renal tubules, in addition to blood vessels (Figure 1). LVD was significantly higher among the severe compared with the stable group (Figure 2). Cases in which the Banff i score indicated higher degrees of mononuclear cell interstitial inflammation showed greater lymph vessels expression (Figure 3). Because all of the other Banff scores were 0, no comparisons could be performed for these examinations. Table 2 shows the real numbers or scores for lymphatic vessels or Banff scores.

DISCUSSION

We examined the expression of lymph vessels after treatment of an ARE, showing that patients in whom renal function showed a 20% increase of s-Cr displayed greater expression of lymph vessels upon re-biopsy. The expression of lymph vessels was related to interstitial cell infiltration in terms of the Banff i score. The cases in which renal function was stable after treatment displayed little expression of lymph vessels around renal tubules.

There are several reports of the relationship between interstitial cell infiltration and the expression of lymph vessels in renal allograft specimens during an ARE.[1–4] The expression of lymph vessels has been immediately recognized in inflammatory cell invasion.[2] Although it may be a speculation because examined specimen were only obtained after treatment, we considered that specimens from cases in which inflammation had disappeared would also show disappearance of lymph vessel, and that specimens showing persistence of inflammation would also show persistence of lymph vessel expression. Stuht et al.[4] reported that interstitial cell infiltration without lymphatic neoangiogenesis was related to renal function degeneration upon protocol biopsies. This observation contradicts our result; however, the difference may depend on the fact that we used episode biopsies, whereas they used protocol biopsies.

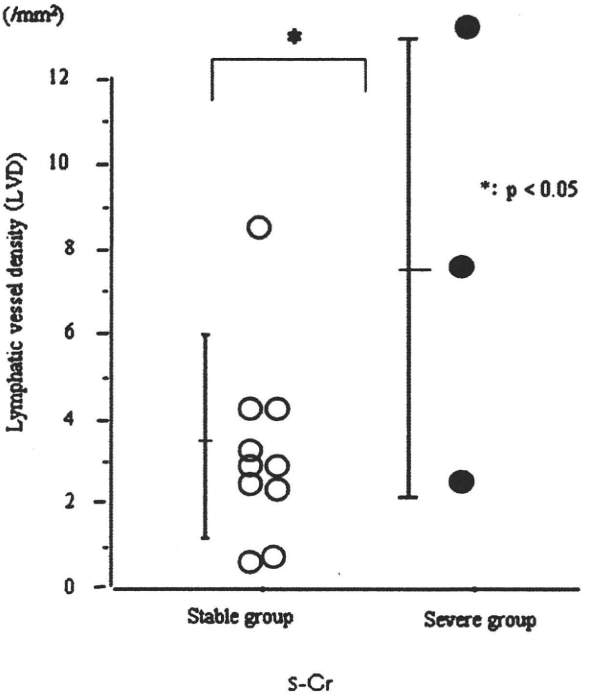

Fig 2. Comparison of lymph vessel density between the stable group and the severe group.

In conclusion, the expression of lymph vessels in renal allograft specimens after treatment of an ARE was related to deterioration of renal function and inflammatory cell invasion. We plan to examine the relation of lymph vessel expression to long-term prognosis.

REFERENCES

1. Kerjaschki D, Regele HM, Moosberger I, et al: Lymphatic neoangiogenesis in human kidney transplants is associated with immunologically active lymphocytic infiltrates. J Am Soc Nephrol 15:6032, 2004

2. Yamamoto I, Yamaguchi Y, Yamamoto H, et al: A pathological analysis of lymphatic vessels in early renal allograft. Transplant Proc 38:3300, 2006

3. Colvin R. Emphatically lymphatic: J Am Soc Nephrol 15:327, 2004

4. Stuht S, Gwinner W, Franz I, et al: Lymphatic neoangiogenesis in human renal allografts: results from sequential protocol biopsies. Am J Transplant 7:377, 2007