Original Research
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Correlation between urine macrophage migration inhibitory factor (MIF)/creatinine ratio and time after kidney transplantation

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Abstract

Background: Despite the long-standing association of macrophage migration inhibitory factor (MIF) with delayed-type hypersensitivity response, the potential role of MIF in chronic allograft nephropathy is unknown. The association between up-regulation of MIF expression, macrophage and T cell infiltration and the severity of chronic allograft nephropathy suggests that MIF may be an important mediator in the process of chronic allograft nephropathy. Therefore, the aims of this study were to measure urine concentration of MIF after renal transplantation, and to determine if it increases with time.

Methods: In this prospective cross-sectional study twenty-two pediatric patients (case, group A) who received kidney transplants between 1999 and 2006, and forty healthy children (control, group B) were recruited. Urine MIF and creatinine were assessed in all patients. Urine MIF concentrations were quantitated by ELISA.

Results: The mean ratios of urine MIF/Creatinine (Cr) were calculated as 5.046(SEM=2.04) pg/μmol creatinine in transplanted-kidney patients (group A) and 1.85(SEM=0.35) pg/μmol creatinine in healthy individuals (group B). A good significant correlation was seen between urine MIF/Cr ratio and time after kidney transplantation in recipients ($P=0.002$, $r_{Spearman} = +0.633$).

Conclusion: This study shows significant correlation between urine MIF/Cr ratio and time passed after transplantation. Increasing MIF/Cr ratios were seen in patients with a longer post transplantation period. Therefore, it is necessary to determine the role of macrophages in chronic renal nephropathy especially chronic rejection with additive studies and then study the effect of anti-MIF antibodies in the treatment of this condition.

Keywords: Chronic allograft nephropathy, macrophage migration inhibitory factor (MIF), creatinine, transplantation.

Introduction

Macrophage migration inhibitory factor (MIF), a 12.5 kDa protein with multiple proinflammatory properties, is considered to be the first "cytokine" discovered, and it was identified initially for its ability to inhibit the random migration of macrophages in culture [1,2]. MIF was described originally to be a product of activated T cells, but the protein is now known to be produced by a variety of mesenchymal, parenchymal and epithelial cell types [3]. MIF is widely expressed and secreted in response to

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inflammatory stimuli, and acts as a counterregulator to the effects of endogenous glucocorticoids [2]. It plays an unexpectedly important role in delayed-type hypersensitivity and the effector phase of immune-mediated injury. MIF activity is required for the phenotypic expression of disease in animal models of glomerulonephritis, arthritis, letal endotoxemia and inflammatory bowel disease [2,4-6]. MIF has subsequently been implicated in macrophage activation and in antigen-driven T cell responses [4,7]. Additionally, it is shown that MIF blockade affects the effector phase of the macrophage-mediated injury through prevention of macrophage activation or function within the target organ [8].

On the other hand, macrophage accumulation has long been recognized as a feature of allograft rejection [9]. The events of chronic rejection seem to be mediated primarily by macrophages and their products. Recent advances in macrophage biology have allowed a better understanding of the mechanisms of macrophage accumulation, their state of activation and the pleuripotent roles they play in allograft rejection. Therefore, these findings raised the possibility that MIF may also be a relevant mediator of allograft rejection. Circumstantial evidence corroborating this possibility includes elevated expression of MIF in rat renal allografts undergoing rejection versus isograft controls and detection of MIF protein associated with mononuclear infiltrates in renal biopsy of specimens of human kidney transplants with rejection [10,11].

Some other studies have demonstrated that macrophage infiltration correlates inversely with renal graft function and is the only best predictor of renal graft survival [12-15]. Furthermore, renal MIF expression is up-regulated in association with macrophage infiltration in experimental models of immunologic kidney disease [16-18]. On these bases, it is postulated that the cytokine macrophage migration inhibitory factor (MIF) plays a pivotal role in the process of renal allograft rejection.

Therefore, the aims of this study were to measure urine concentration of MIF after renal transplantation, to determine if it increases with time after transplantation.

Methods

In this prospective cross-sectional study twenty-two pediatric patients (case, group A) who received kidney transplants between 1999 and 2006, and forty healthy children (control, group B) were recruited. In the case group (group A), prednisolone was administered in a dose of 250 mg for the first 3 days after transplantation and then reduced to 200 mg, 150 mg, 100 mg, and 60 mg during the next 4 days, followed by 40 mg/day for 3 days, 30 mg/day for 3 days, and 20 mg/day. This dose was then reduced by 0.15 mg/kg every 15–25 days. The maintenance dose of prednisolone was 0.15 mg/kg every other day as long as the graft survived. Cyclosporine was administered at 12 mg/kg per day for the first 10 days, and was then reduced by 2 mg/kg every 10 days until a dose of 4–6 mg/kg per day was reached. At that stage, dosage was adjusted to maintain a target 24-h trough level of 100–150 ng/ml during the 1st month and 80–100 ng/ml thereafter. Mycophenolate mofetil was administered at 600 mg/m^2 every 12 h. All case group patients (group A) received transplants from live donors.

To determine urinary macrophage migration inhibitory factor (MIF) concentration, urine samples of both transplanted patients and healthy volunteers were collected for analysis. The concentration of MIF in urine samples was quantitated by enzyme-linked immunosorbent assay (ELISA) and corrected for urine creatinine. Furthermore, the last serum creatinine, time after transplantation and undergoing dialysis were determined in transplanted children of group A. No patients had evidence of urinary tract infections during this period and also, there were no significant differences in age and gender distribution between these case and con-

trol groups.

Urine samples

Sterile midstream urine samples were collected and stored at 4°C for a maximum of 6 hr before processing. The urine was centrifuged at 1500×g for 10 min to separate debris and then a protease inhibitor cocktail (Sigma, Castle Hill, NSW, Australia) was added (5μl/ml) before storage at -80°C. A 1-ml aliquot was analyzed for urine creatinine.

MIF ELISA

Urine MIF concentrations were quantitated by ELISA according to the manufacturer's instructions (R & D Systems, Minneapolis, MN). In brief, ELISA plates were coated overnight with 2 μg/ml mouse anti-human MIF capture antibody. Wells were washed with 0.05% Tween-20 PBS (PBST) and then blocked with 5% sucrose, 1% bovine serum albumin (BSA), 0.05% NaN_3 in PBS for 2 hr. Test samples (human urine) were diluted in 0.1% BSA, 0.05% Tween-20 in 20 mM Tris-HCl, 150 mM NaCl, pH 7.3. After washing, samples were incubated with 1.25 ng/ml peroxidase-conjugated streptavidin (Zymed, South San Francisco, CA) for 30 min, washed in PBST, and then incubated for 30 min with 100 μl/well ready to use TMB (3,3`,5,5;-tetramethylbenzidine) (Zymed) and the colorimetric reaction stopped by the addition of 0.5 M H_2SO_4. Finally, the adsorption at 450/570 nm was measured using a microplate reader.

Statistical methods

Data were analyzed using SPSS v.13 software. Descriptive results are expressed as the mean ± SEM or SD. In order to compare urine MIF, creatinine and their ratios in two groups, Mann-Whitney U-test and Independent t-test were performed. Pearson and Spearman correlations were also used to evaluate the relationship between quantitative variables. Receiver operating curve (ROC) analysis was performed to assess the predictability of chronic allograft nephropathy and time post-transplantation with quantitative variables of the study, and then to compare area under curve (AUC) of these variables. All P-values were two-tailed and $P<0.05$ was considered statistically significant.

Results

Twenty-two transplanted-kidney patients and 40 healthy individuals were recruited in groups A (Case) and B (Control), respectively. All transplanted kidneys were from living unrelated donors.

The mean age of 22 patients in group A was 12.91(SD=2.22) years (range 9-17) with 14 (63.6%) males and 8(36.4%) females, and the mean age of individuals in group B was 11.63

No. of Patients	22
Gender (male/female)	14/8
Age (mean±SD)	12.91±2.22 yr (9-17)
Primary disease	
Hypo/dysplastic kidney	6
Medullary cystic kidney disease	4
Reflux nephropathy	4
others	8
Pretransplant dialysis	
Yes	7
Pre-emptive	15
Last serum creatinine (mean±SD)	1.19±0.41mg/dl(0.7-2.5)
Time after transplantation (mean±SD)	3.91±1.85 yr (1-7)

Table 1. Characteristics of the patients (Group A).

	A (Case)	B (Control)	P value
Age *(year)Mean(±SD)*	12.91(±2.22)	11.63 (±1.85)	0.756
Gender	Male:14 (63.6%) Female:8 (36.4%)	Male:25 (62.5%) Female:15 (37.5%)	0.929
Urine Cr *(mg/dl)Mean(±SEM)*	172.95 (±18.96)	172.63 (±11.51)	0.924
Urine MIF *(pg)Mean(±SEM)*	821.50 (±364.67)	338.83 (±67.30)	0.843
Urine MIF/Cr ratio *(pg/µmol creatinine)Mean(±SEM)*	5.046 (±2.04)	1.85 (±0.35)	0.137

Table 2. Comparison of the demographic and main study variables in the two groups.

(SD=1.85) years (range 8-16) with 25 (62.5%) males and 15 (37.5%) females.

More demographic characteristics of patients are listed in Table 1. As it is shown, the mean time after transplantation was 3.91 (SD=1.85) years (range 1-7) and the mean of last serum creatinine was 1.19 (SD=0.41) mg/dl (range 0.7-2.5).

The mean levels of urine creatinine in groups A and B were 172.95(SEM=18.96) and 172.63 (SEM=11.51) mg/dl, respectively. In addition, the mean ratios of urine MIF/Cr were calculated as 5.046 (SEM=2.04) pg/µmol creatinine in transplanted-kidney patients (group A) and 1.85 (SEM=0.35) pg/µmol creatinine in healthy individuals (group B). Although urine MIF/Cr ratio was greater among the patients, the difference was not statistically significant (P>0.05, Power = 56%, Table 2).

However, retrospectively evaluation of data demonstrated a good significant correlation between urine MIF/Cr ratio and time after kidney transplantation in recipients (P=0.002, $r_{Spearman}$ = +0.633, Fig. 1). It is suggested that the more it passes from the time of transplantation, the greater the ratio of urine MIF/Cr will become. Moreover, a significant direct correlation was also found between urine MIF and time after kidney transplantation (P<0.001, $r_{Spearman}$= +0.693).

Whereas, no significant correlation was found between serum creatinine and time after kidney transplantation among recipients (P>0.05).

As shown in Table 3, more detailed analysis was performed in group A. It demonstrated that even a more notable significant correlation ex-

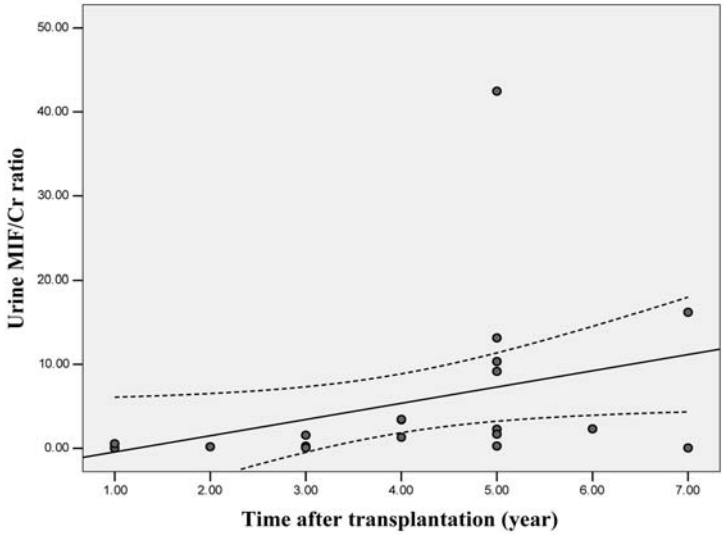

Fig. 1. Correlation between urine MIF/Cr ratio and time after transplantation.
(P=0.002, $r_{Spearman}$ = +0.633)

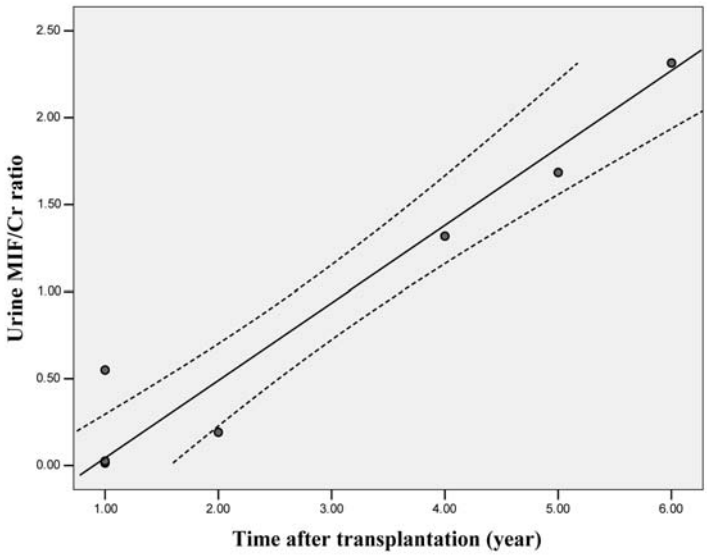

Fig. 2. Correlation between urine MIF/Cr ratio and time after transplantation in patients who underwent pretransplant dialysis (P=0.000, $r_{Pearson}$ = +0.964).

ists between urine MIF/Cr ratio and time after kidney transplantation in those who underwent pretransplant dialysis by retrospective observational study of these data (P=0.000, $r_{Pearson}$ = +0.964, Fig. 2). But the data in Table 3 revealed that the difference between urine MIF/Cr ratio in pre-emptive and dialyzed recipients is not statistically significant [6.99 (SEM=2.87)] pg/µmol creatinine vs. 0.87 (SEM=0.34) pg/µmol creatinine, P>0.05, Power = 33%)].

On the other hand, the differences between urine MIF/Cr ratio and urine MIF level in recipients with time after transplantation of >3 years and those with ≤ 3 years were statistically significant (P= 0.028 and P= 0.000, respectively). As listed in Table 3, the mean urine MIF/Cr ratio in patients with time after transplantation of >3 years was significantly greater than those with time after transplantation of ≤ 3 years [7.73 (SEM=3.00) pg/µmol creatinine vs. 0.34 (SEM=0.19) pg/µmol creatinine, P= 0.028]. In comparison the mean urine MIF/Cr ratio in the control group was 1.85 (SEM=0.35) pg/µmol creatinine. This showed that the ratio was significantly different between control group and patients with time after renal transplantation of >3 years, but not in patients with time after transplantation of ≤ 3 years.

	No. of patients	Urine Cr (mg/dl) Mean (±SEM)	Urine MIF (pg) Mean (±SEM)	Urine MIF/Cr ratio (pg/µmol creatinine) Mean (±SEM)	Serum Cr (mg/dl) Mean(±SEM)
Time after transplantation					
≤ 3 years	8	159.75 (±29.86)	50.38 (±31.95)	0.34 (±0.19)	1.22 (±0.20)
> 3 years	14	180.50 (±25.03)	1262.14 (±544.24)	7.73 (±3.00)	1.18 (±0.08)
P value		0.611	0.000*	0.028*	0.805
Pretransplant dialysis					
Dialysis	7	184.29 (±34.02)	213.29 (±110.03)	0.87 (±0.34)	1.19 (±0.22)
Pre-emptive	15	167.67 (±23.52)	1105.33 (±521.60)	6.99 (±2.87)	1.20 (±0.08)
P value		0.693	0.123	0.052	0.942

Table 3. Comparison of the results in different patient subgroups (Group A).

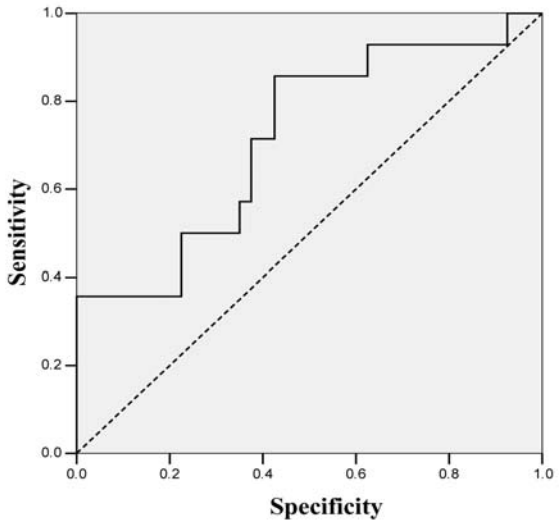

Fig. 3. Receiver operating curve (ROC) for urine MIF/CR ratio in patients with time after transplantation of more than 3 years (P= 0.016, Area under curve= 0.718).

Additionally, the mean level of urine MIF in recipients with time after transplantation of >3 years was 1262 (SEM=544.24) pg, while it was 50.38 (SEM=31.95) pg in those with time after transplantation of $\leq$ 3 years (P=0.000).

By the way, no statistically significant difference was observed between the last level of serum creatinine in these subgroups (P>0.05).

In addition, Receiver Operating Curve (ROC) analysis was performed in this study and demonstrated that in recipients with time after transplantation of >3 years, urine MIF/Cr ratio could be considered a potentially useful index to evaluate chronic allograft nephropathy of the transplanted kidney (P=0.016, Area under curve = 0.718, Fig. 3).

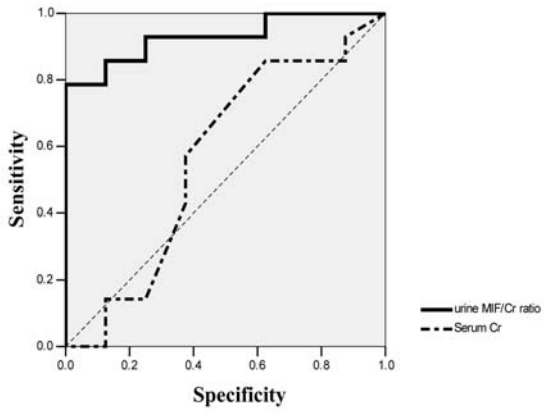

Fig. 4. Comparison of the ROC's for urine MIF/Cr ratio and serum creatinine to predict time after transplantation of >3 years in recipients (urine MIF/Cr ratio: P = 0.001, AUC = 0.929 vs. serum Cr: P = 0.609, AUC = 0.567).

Also it was shown that urine MIF/Cr ratio has greater AUC than serum creatinine to predict time after transplantation of >3 years in recipients ($AU_{Curine\ MIF/Cr}$ = 0.929 vs. $AUC_{serum\ Cr}$ = 0.567, Fig. 4).

Discussion

MIF is a proinflammatory cytokine that was originally described as a product of activated lymphocytes that inhibited the random migration of guinea pig peritoneal macrophages in vitro and promoted macrophage accumulation in the delayed-type hypersensitivity response [1,19]. MIF has a lot of important immune functions in addition to the recruitment of macrophages including increasing HLA-DR expression on macrophages, T cell activation, augmentation of IL-2-driven T cell proliferation, stimulation of T cell-dependent antibody production, and activation of macrophages by increasing nitric oxide production [7, 20].

By the way, chronic graft nephropathy has been defined as progressive functional deterioration, occurring months to years after grafting [21-23].

MIF mRNA and protein is constitutively expressed in normal kidney, being largely restricted to tubular epithelial cells, some glomerular epithelial cells, and vascular smooth muscle cells. In both acute and chronic renal allograft rejection, there was marked up-regulation of MIF mRNA and protein expression by intrinsic kidney cells such as tubular epithelial cells and vascular endothelial and smooth muscle cells. There was also MIF expression by infiltrating macrophages and T cells. Of note, macrophage and T cell infiltrates were largely restricted to areas with marked up-regulation of MIF expression, potentially contributing to the development of severe tubulitis and intimal or transmural arteritis [10]. Beckmann et al evaluated

the detection of iron-loaded macrophages at magnetic resonance (MR) imaging as a noninvasive means to monitor early signs of chronic allograft rejection in the life-supporting Fisher-to-Lewis rat kidney transplantation model. They found the stages of chronic rejection as follows: A decrease in cortical MR signal intensity occurred in allografts between 8 and 16 weeks after transplantation (due to iron loaded macrophages). Proteinuria occurred at 16 weeks. Blood and urine creatinine levels remained unchanged up to week 28 [24].

We found a significant correlation between urine MIF/Cr ratio and the time passed from transplantation. A longer duration of transplantation was related with a higher MIF/Cr ratio. Whereas, we did not find a relationship between the last serum creatinine of patients and time after renal transplantation. On the other hand, the differences between urine MIF/Cr ratio and urine MIF level in recipients with time after transplantation of >3 years and those with $\leq$ 3 years were statistically significant. It is believed that macrophage infiltration increases by time after transplantation, especially after 3 years from transplantation.

To the best of our knowledge, our work is the first to evaluate the association between MIF/Cr ratio and time after kidney transplantation in humans. We showed that urine MIF/Cr ratio increases progressively after transplantation. However, as renal biopsies were not obtained in our study, this can be due to all causes of chronic renal nephropathy with macrophage and T cells as key cells in pathogenesis of them such as chronic rejection and cyclosporine toxicity. More studies are needed to show the importance of serial urine MIF/Cr ratio measurements in renal transplantation in early recognition of chronic renal damage.

In other words, despite not having taken renal biopsies in our study, increasing urine MIF/Cr ratio with time after transplantation could possibly indicate increasing macrophage infiltration after kidney transplantation; which itself could have probable association with chronic allograft nephropathy (CAN), as macrophages play the main role in the pathogenesis of CAN. However, it is necessary to perform some more research on this topic with larger sample sizes and taking renal biopsies if possible.

In our opinion it is necessary to determine the role of macrophages in chronic renal nephropathy especially chronic rejection with additive studies and then the effect of anti-MIF antibodies in the treatment of this condition. This therapeutic attention to macrophages, in addition to T lymphocytes, may lead to improved outcomes in organ transplantation.

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