Plasma Homocysteine and Glutathione Are Independently Associated With Estimated Glomerular Filtration Rates in Patients With Renal Transplants

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ABSTRACT

Background. Elevated levels of plasma homocysteine could, through homocysteine oxidation, induce the overproduction of reactive oxygen species, leading to a reduction in glutathione-related antioxidants, and may impair graft functions in patients with renal transplants. The purpose of this study was to determine whether plasma homocysteine, glutathione, or its related antioxidants were related to graft functions in patients with renal transplants.

Patients and Methods. We recruited 66 patients (mean age 48.4 years) with renal transplants (mean transplant duration 8.3 years). Patients were divided into 2 groups, based on their estimated glomerular filtration rate (eGFR): the moderate graft function group (eGFR $\geq$ 60 mL/min/1.73 m^2, n = 37) and low graft function group (eGFR $<$ 60 mL/min/1.73 m^2, n = 29). We then determined their fasting levels of the following: malondialdehyde (MDA), homocysteine, cysteine, pyridoxal 5′-phosphate (PLP), glutathione (GSH), oxidized glutathione (GSSG), GSH/GSH ratio, glutathione peroxidase (GSH-Px) activity.

Results. We found in the low graft function group significantly higher levels of plasma homocysteine, cysteine, GSH, and GSH/GSSG ratios. But an intergroup difference was not found regarding levels of MDA, PLP, GSSG, and GSH-Px activity. After adjusting for potential confounders, the increased plasma homocysteine and GSH levels were independently associated with lower eGFR. No interaction existed between homocysteine and GSH levels in association with eGFR.

Conclusion. Increased plasma homocysteine and GSH levels appeared to be independent indicators of decreased graft functions in patients with renal transplants.

CHRONIC graft dysfunction is a challenge for maintaining long-term survivals of renal transplant recipients. Hyperhomocysteinemia [1,2] is associated with the loss of graft functions in patients receiving renal transplants. Increased homocysteine levels could induce overproduction of free radicals through homocysteine oxidation, resulting in oxidative stress [3,4]. In the homocysteine metabolism, homocysteine is converted, through transsulfuration by pyridoxal 5′-phosphate (PLP, a vitamin B–6 coenzyme form), to cysteine. Cysteine could then react with glutamate and glycine to generate glutathione (GSH). GSH is oxidized to glutathione disulfide (GSSG, oxidized form of GSH). Along with GSH peroxidase (GSH-Px) and GSH

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S-transferase, these molecules play a fundamental role in cellular defense against free radicals coming from oxidative stress. High levels of GSH are present in the kidney, particularly in the proximal tubules, to maintain normal renal functions [5]. GSH and its related antioxidant enzymes could be depleted in order to cope with increased oxidative stress via homocysteine accumulation. Alternatively, GSH synthesis could be reduced along with the progressive decline of graft function. Previous studies showed that both plasma GSH concentration and GSH-Px activities dropped with reduced renal functions [6,7]. Even though GSH and GSH-Px activities increased after renal transplantation, these increases did not reach the normal levels seen in healthy subjects [8].

Both the increase in homocysteine concentration and the decrease in GSH and GSH-related antioxidant capacities were associated with the loss of graft renal functions. It remains unclear if these 2 changes act independently or interact with each other during the functional loss. To this end, we have determined whether plasma homocysteine, GSH, or GSH-related antioxidant capacities act dependently or independently during the loss of graft function in patients with renal transplants.

PATIENTS AND METHODS

This was a cross-sectional study. Patients were Taiwanese subjects, aged $\geq$ 20 and $<$ 80 years old, who underwent kidney transplantation and were regularly followed up by a nephrologist at the outpatient clinic of the Division of Nephrology, Taichung Veterans General Hospital. Most patients received a maintenance dose of triple-therapy immunosuppression. Patients excluded were those clinically unstable; pregnant; lactating; or having cardiovascular disease, liver disease, chronic inflammatory disease, cancer, or alcoholism. Patients were classified, based on their graft function, into 1 of the following groups: moderate graft function group (estimated glomerular filtration rate [eGFR] $\geq$ 60 mL/min/1.73 m^2) or low graft function group (eGFR $<$ 60 mL/min/1.73 m^2). This study was approved by the Institutional Review Board of Taichung Veterans General Hospital. Each subject provided informed consent prior to participation.

We recorded personal data of each patient such as age, sex, smoking and drinking habits, along with their use of medications and medical history. Their height, weight, and systolic and diastolic blood pressures were also measured. Body mass index (kg/m^2) was calculated. Fasting blood samples were drawn in vacutainer tubes containing anticoagulant or no anticoagulant. Serum or plasma was separated within 30 minutes after blood collection and analyzed immediately, if not frozen (−80°C) and analyzed later. Serum albumin, hemoglobin, creatinine, and blood urea nitrogen were measured using an automated biochemical analyzer. Plasma malondialdehyde (MDA) concentration was determined with thiobarbituric acid-reactive substances to indicate the level of oxidative stress [9]. Plasma PLP [10], homocysteine, and cysteine [11] were quantified by high-performance liquid chromatography using a fluorescence detector. Hyperhomocysteinemia was defined as plasma homocysteine concentrations $\geq$ 14 mmol/L [12]. Plasma GSH and GSSG concentrations, along with GSH-Px activity, were determined using respective commercial kits (Cayman Chemical Company, Ann Arbor, MI, USA). All analyses were performed in duplicates.

All data analyses were performed using the SAS statistical software package (version 9.4; Statistical Analysis System Institute Inc., Cary, NC, USA). Normal distribution was assessed with the Shapiro-Wilk test. Student *t* test or Mann-Whitney U test was used to determine statistically the intergroup differences on anthropometric measurements and biochemical values. For categorical variables, the differences were assessed by the χ^2 or Fisher's exact test. The Spearman correlation coefficient (r_s) was used to assess the relationship between oxidative stress indicator (MDA level) and homocysteine or GSH level. Multiple linear regression was used to evaluate the associations of the levels of homocysteine, cysteine, GSH, GSSG and GSH-Px, and the eGFR level (dependent variable), after adjusting for age, sex, body mass index, diabetes, cardiovascular disease, drinking and smoking habits, and transplantation duration. Results were considered statistically significant at $P < .05$. Values presented in the text are means ± standard error of mean.

RESULTS

We found 37 patients with moderate graft function and 29 patients with low graft function. Demographic and clinical characteristics are listed in Table 1. Recipients with moderate graft function had significantly higher levels of eGFR and hemoglobin and lower levels of serum creatinine and blood urea nitrogen.

Table 2 shows the moderate graft function group having significantly lower levels of homocysteine, cysteine, GSH, and GSH/GSSG ratio. Patients had hyperhomocysteinemia ($\geq$ 14 μmol/L) in the low graft function groups, and such patients were twice as many compared with the moderate graft function group. Meanwhile, we found no significant intergroup differences in the levels of MDA, PLP, GSSG, and GSH-Px activity.

Table 1. Demographic and Clinical Characteristics of Renal Transplant Recipients

Characteristics	Moderate Graft Function Group (n = 37)	Low Graft Function Group (n = 29)
eGFR (mL/min/1.73 m^2)	78.24 ± 2.49	42.60 ± 2.22*
Age (y)	47.89 ± 1.73	49.00 ± 2.35
Sex (male/female)	22/15	15/14
Body mass index (kg/m^2)	23.89 ± 0.73	24.03 ± 0.84
Transplantation duration (y)	7.19 ± 0.75	9.69 ± 1.20
Blood pressure (mm Hg)		
Systolic	130.27 ± 2.09	136.03 ± 3.37
Diastolic	82.78 ± 1.47	81.66 ± 1.90
Serum albumin (g/dL)	4.30 ± 0.05	4.26 ± 0.07
Serum hemoglobin (g/dL)	13.81 ± 0.32	12.51 ± 0.33*
Serum creatinine (mg/dL)	1.00 ± 0.03	1.69 ± 0.11*
Blood urea nitrogen (mg/dL)	16.97 ± 0.81	27.69 ± 2.70*
Current smoking habits (n, %)	2, 5.41%	4, 13.79%
Current drinking habits (n, %)	6, 16.22%	1, 3.45%
Comorbidities (n,%)		
Diabetes Mellitus	13, 35.14%	7, 24.14%
Cardiovascular disease	3, 8.11%	4, 13.79%

Values are means ± standard error of mean.
*Value is significantly different from the other group; $P < .05$.

Table 2. Biochemical Measurements of Renal Transplant Recipients

Characteristics	Moderate Graft Function Group (n = 37)	Low Graft Function Group (n = 29)
Malondialdehyde (μmol/L)	0.73 ± 0.03	0.67 ± 0.03
Homocysteine (μmol/L)	14.21 ± 0.76	18.69 ± 1.31*
> 14 μmol/L (n, %)	14, 37.84%	23, 79.31%
Cysteine (μmol/L)	219.23 ± 5.95	248.47 ± 8.49*
Pyridoxal 5′-phosphate (nmol/L)	76.70 ± 12.41	54.98 ± 6.56
Glutathione (μmol/L)	95.98 ± 3.78	128.67 ± 10.64*
Glutathione disulfide (μmol/L)	668.52 ± 7.23	681.56 ± 19.91
GSH/GSSG ratio	0.14 ± 0.01	0.18 ± 0.01*
GSH-Px activity (nmol/mL/min)	134.44 ± 9.07	154.31 ± 22.56

Values are means ± standard error of mean.
Abbreviations: GSH, glutathione; GSSG, glutathione disulfide; GSH-Px, glutathione peroxidase.
*Value is significantly different from the other group; $P < .05$.

Significant and negative associations existed between eGFR values and levels of homocysteine and GSH in all patients (n = 66) after adjusting for potential confounders. However, no such associations were found between the levels of eGFR and GSSG or GSH-Px activity (Table 3). Also, no interactions were found between levels of plasma homocysteine and GSH in association with eGFR (Table 3). A significant association was found between MDA and GSH levels in all patients ($r_s = 0.24$, $P < .05$).

DISCUSSION

In our previous study [13], high homocysteine concentration was found to be an independent factor in the development of early stage chronic kidney disease (CKD) (stage 2 and 3). Consistent with that, our present results also indicated a higher percentage of hyperhomocysteinemia in patients with low graft function and increased plasma homocysteine concentrations following a decrease in graft function. Although the cause-effect relationship between plasma homocysteine and renal function cannot be determined in the present cross-sectional study, a strategy of reducing plasma homocysteine might be beneficial to prevent the loss of graft function after the renal transplantation.

In our patients, the accumulation of homocysteine in plasma did not seem to affect its trans-sulfuration metabolism, and the GSH concentration increased concomitantly with the loss of graft functions. In GSH biosynthesis, cysteine is a major, and limiting, substrate. Although plasma homocysteine had increased following the loss of graft function, cysteine also increased, but that did not affect GSH biosynthesis in patients with low graft function. Since increased oxidative stress is an important metabolic sequela in patients with CKD [14,15], changes in the GSH antioxidant defense system might alter the status of oxidative stress. Although we had not measured the activity of GSH reductase, we assumed that GSH reductase activities of patients with low graft function remained adequate to the

Table 3. Multiple Linear Regression With Renal Function as Dependent Variable and Clinical Parameters and Biochemical Measurements as Independent Variables in Renal Transplant Recipients

	eGFR (dependent variable)	
Parameters	Covariate Model	β (Standard Error)
Homocysteine	Confounders§	-2.03 (0.49)‡
	GSH + confounders	-1.93 (0.46)‡
	GSH + homocysteine × GSH + confounders	-0.98 (1.52)
	GSSG + confounders	-2.12 (0.49)‡
	GSSG + homocysteine × GSSG + confounder	5.10 (5.08)
	GSH/GSSG ratio + confounders	-1.77 (0.46)‡
	GSH/GSSG ratio + homocysteine × GSH/GSSG ratio+ confounder	-0.47 (1.69)
	GSH-Px + confounders	-2.12 (0.49)‡
	GSH-Px + homocysteine × GSH-Px + confounder	-1.79 (1.19)
GSH	Confounders	-0.20 (0.07)†
	homocysteine + confounders	-0.18 (0.06)†
	homocysteine + GSH × homocysteine + confounders	-0.02 (0.24)
GSSG	Confounders	-0.03 (0.04)
	homocysteine + confounders	-0.05 (0.04)
	homocysteine + GSSG × homocysteine + confounders	0.13 (0.13)
GSH/GSSG ratio	Confounders	-216.85 (63.40)†
	homocysteine + confounders	-174.87 (57.90)†
	homocysteine + GSH/GSSG ratio × homocysteine + confounders	-40.33 (177.88)
GSH-Px activity	Confounders	-0.02 (0.03)
	homocysteine + confounders	-0.04 (0.03)
	homocysteine + GSH-Px × homocysteine + confounders	0.00 (0.14)

Abbreviations: N = 66. β, regression coefficient; eGFR, estimated glomerular filtration rate; GSH, glutathione; GSSG, glutathione disulfide; GSH-Px, glutathione peroxidase.
†$P < .01$.
‡$P < .001$.
§Adjusted for age, sex, body mass index, diabetes mellitus, cardiovascular disease, smoking and drinking habits, and transplantation duration.

extent that GSSG was properly reduced to GSH as we found higher ratios of GSH/GSSG in the low graft function group. As a consequence, GSH levels could be kept high in the low graft function group to cope with increased oxidatrive stress via the accumulation of homocysteine. Those with low graft function could thus maintain a similar oxidative stress status when compared to those with moderate graft function. However, in the reported studies, unlike changes of plasma GSH, along with the loss of renal function in other CKD patients, their erythrocyte GSH concentrations could be either elevated [16], decreased [17,18] or remained unaltered [19]. We cannot rule out the possibility that the utilization and turnover of plasma GSH is redistributed from erythrocytes to plasma, leading to elevated plasma GSH levels during the loss of renal function. While the distribution patterns of plasma and erythrocyte GSH need to be clarified, the plasma level of GSH appeared to vary independent of homocysteine concentrations in relation to graft function.

The main limitation of this study is cross-sectional design with only 1 time point in measuring all biochemical variables, and the cause-effect relationship failed to be established. In addition, a larger sample size shall strengthen the statistical power in revealing a definitive association between eGFR and GSH status in moderate and low graft function groups.

In conclusion, increased plasma homocysteine and GSH levels appeared as independent indicators of decreased graft function in renal transplant patients.

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