Effect of Renal Transplantation on Biomarkers of Inflammation and Oxidative Stress in End-Stage Renal Disease Patients

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Background. Chronic kidney disease patients have a high prevalence of inflammation and oxidative stress, and this has been associated with the excess cardiovascular morbidity and mortality observed in this population. Because maintenance hemodialysis is ineffective in controlling these factors, we hypothesized that restoration of kidney function by transplantation would be required to improve uremic inflammation and oxidative stress.
Methods. This was a prospective cohort study evaluating time-dependent changes in biomarkers of inflammation and oxidative stress before and after renal transplantation. Nineteen end-stage renal disease (ESRD) patients (age 38.3±13.7 years, 58% male, 95% white, 21% diabetic) undergoing living-donor renal transplantation were enrolled. C-reactive protein (CRP), interleukin (IL)-6, IL-10, tumor necrosis factor (TNF)-α, protein-associated carbonyl content, and F_2-isoprostanes were assessed at 1 week pretransplantation and at 1 week and 2 months posttransplantation.
Results. Pretransplant levels of the pro-inflammatory proteins IL-6, TNF-α, and CRP, as well as the oxidative stress markers plasma protein carbonyls and F_2-isoprostanes, were significantly elevated in ESRD patients compared with healthy control subjects. We observed rapid and significant declines in all of these biomarkers after transplantation that persisted for 2 months.
Conclusions. Our findings indicate that restoration of renal function by transplantation improves the chronic inflammation and increased oxidative stress associated with uremia, which may contribute to the improved survival afforded to ESRD patients by renal transplantation.

Keywords: Kidney transplantation, Cytokines, C-reactive protein, Inflammation, Oxidative stress.

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Despite recent advances in our understanding of the uremic state and improvements in the science and technology of renal-replacement therapies, the prognosis of patients with end-stage renal disease (ESRD) remains poor, with an annual cardiovascular mortality rate that is 10- to 20-fold greater than the general population (*1*). Although there is generally an over-representation of the classical cardiovascular risk factors, such as hypertension, diabetes mellitus, dyslipidemia, left ventricular hypertrophy, and heart failure, among patients with chronic renal failure, these alone fail to account for the substantial burden of atherosclerotic disease and the poor clinical outcomes common to this patient group (*2*). Data from recent epidemiologic studies demonstrate that even moderate reductions in kidney function (beginning at a glomerular filtration rate [GFR] of 60 mL/min) are associated with increased cardiovascular risk and that the level of kidney function itself is an independent predictor of cardiovascular outcomes and all-cause mortality (*3*, *4*), supporting the premise that other factors related to the uremic environment per se accelerate atherosclerosis.

Among these nontraditional risk factors, inflammation and oxidative stress have been proposed as mechanistic links between chronic uremia and cardiovascular disease (CVD) (*5*, *6*). Inflammation of the vessel wall and oxidative modification of low-density lipoproteins are now recognized as key events in the early development of atherosclerosis (*7*, *8*). Indeed, numerous studies document that elevated levels of the pro-inflammatory cytokine interleukin (IL)-6 and the acute

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phase reactant C-reactive protein (CRP) are important predictors of cardiovascular events, both in the general population and in patients with chronic kidney disease (CKD) (*9–12*) and that circulating concentrations rise as renal function deteriorates. Likewise, high levels of oxidative stress markers are also common in CKD patients and have been correlated with the occurrence and severity of CVD (*13*, *14*).

Conventional dialytic therapies typically are able to lessen uremic symptoms and improve the overall metabolic milieu in patients with ESRD. However, their efficacy in ameliorating the inflammation and oxidant stress observed with loss of kidney function remains controversial. Renal transplantation, the treatment of choice for ESRD, is associated with significantly improved long-term survival compared with that of ESRD patients awaiting transplantation (*15*). Although the reasons underlying this survival advantage are unclear, several theories have been proposed, including better clearance of uremic toxins such as advanced glycosylation end products, regression of left ventricular hypertrophy, and improvement in nutritional status (*16*, *17*). However, there is a paucity of data examining the impact of renal transplantation on the chronic inflammation and oxidative stress commonly observed in uremia and atherosclerosis. We hypothesized that restoration of kidney function by transplantation would improve biomarkers of inflammation and oxidative stress in patients with ESRD. To investigate this, we performed a prospective cohort study in 19 ESRD patients, evaluating time-dependent changes in biomarkers of inflammation and oxidative stress after living-donor renal transplantation.

METHODS

Subjects

Between January 2002 and July 2003, we studied 19 patients with stage 5 CKD (GFR<15 mL/min) undergoing living-donor renal transplantation at Vanderbilt University Medical Center and Maine Medical Center. Patients 16 years of age or older who were approved for transplantation by the transplant nephrology and surgery staffs of each institution were recruited from the outpatient renal-transplant clinics within 2 weeks before their date of surgery. Those with acute illnesses or a previous renal transplant were excluded from the study. Twenty patients consented to participate in the study and were followed for 2 months posttransplantation. Of the participants, 13 (65%) were at Maine Medical Center, and 7 (35%) were at Vanderbilt University Medical Center. One participant was excluded from the final data analysis because of inability of the investigators to collect blood samples in a timely manner because of missed clinic appointments, leaving a final study population of 19 patients. In addition, we recruited 50 nonpregnant healthy subjects with no known CVD or diabetes mellitus from a database of employees of Maine Medical Center Research Institute and Vanderbilt University Medical Center and from an internal medicine practice in Portland, Maine to serve as the control group. The institutional review board of each participating center approved the study protocol, and written consent was obtained from all study subjects.

Information was obtained through clinic interviews, physical examination, laboratory tests, and review of medical records. Blood was collected after an overnight fast for the measurement of a complete blood count and levels of serum urea nitrogen, creatinine, albumin, prealbumin, and inflammatory and oxidative stress biomarkers at the preoperative clinic visit (1 week befor renal transplantation) and at clinic visits 1 week and on average 2 months posttransplantation according to the renal-transplant clinic schedules. We estimated posttransplant GFR using the Modification of Diet in Renal Disease formula (*18*), which is based on patient age, sex, race, serum creatinine, blood urea nitrogen, and albumin levels.

At the time of renal transplantation, all patients underwent induction immunosuppressive therapy with intravenous methylprednisolone (500 mg intraoperatively) and either antithymocyte rabbit immunoglobulin (1 mg/kg intraoperatively and on postoperative days 1, 2, and 4; 14 patients), or basiliximab (20 mg intraoperatively and postoperative day 4; 5 patients), a murine monoclonal antibody directed against the IL-2 receptor, as determined by the transplant team at each center. Maintenance immunosuppression consisted of cyclosporine plus mycophenolate mofetil and prednisone (10 patients), tacrolimus plus mycophenolate mofetil and prednisone (6 patients), tacrolimus plus sirolimus and prednisone (2 patients), or tacrolimus and prednisone (1 patient).

Blood Sampling

Biochemical indices specific to the study included the pro-inflammatory cytokines IL-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α; the anti-inflammatory cytokine IL-10; the acute phase reactant CRP; and plasma protein carbonyl content and plasma free F_2-isoprostanes as markers of oxidative stress. Venous blood was drawn into Vacutainer (Becton-Dickinson, Franklin Lakes, NJ) tubes containing ethyldiaminetetraacetic acid supplemented with 1,000 U/mL catalase and serum separator tubes containing clot activator for plasma and serum separation, respectively. Samples for plasma collection were transported on ice and immediately centrifuged at 4°C at 1,700*g* for 15 minutes, whereas the samples for serum collection were allowed to clot at room temperature before centrifugation. Plasma and serum samples were thereafter stored at −70°C until analysis.

Inflammatory Biomarkers

Cytokine concentrations were measured in duplicate by enzyme-linked immunosorbent assay (ELISA) with kits from BioSource International (Camarillo, CA). IL-1β, IL-6, and TNF-α were measured in plasma and IL-8 and IL-10 in serum. The assay analytical sensitivity was 2.0 pg/mL for Il-1β and IL-6, 3.0 pg/mL for TNF-α, 0.7 pg/mL for IL-8, and 1.0 pg/mL for IL-10. Inter- and intra-assay variability for the cytokine measurements were as follows: 5% and 4% for Il-1β, 6% and 8% for IL-6, 10% and 5% for TNF-α, 5% and 5% for IL-8, and 3% and 4% for IL-10, respectively. Serum CRP levels were measured using the high-sensitivity particle-enhanced immunoturbidimetric assay by Roche Modular System (Indianapolis, IN). Analytical sensitivity of the CRP assay was 0.003 mg/dL.

Plasma Protein Carbonyl Content

Plasma protein carbonyl content measures reactive aldehyde content as an index of oxidative stress. Carbonyl

groups were measured in duplicate using the Zentech PC Test Kit from Zenith Technology (Dunedin, New Zealand). This kit follows the method outlined by Buss et al. (*19*), as amended by Winterbourn and Buss (*20*), which uses derivatization of protein carbonyls in samples and oxidized protein standards with dinitrophenylhydrazine, followed by ELISA with anti-DNP antibody, as we have previously described (*21*). Standard ELISA techniques for labeling and visualizing labeled molecules were used, and absorbance was read at 450 nm on an MRX microplate reader from Dynex Technologies (Chantilly, VA). A standard curve was plotted, and the carbonyl concentration of samples was read off the curve using the MRX Revelation Software. The assay analytical sensitivity was 0.021 nmol/mg protein, and intra-assay variability was 3.8%. The inter-assay variability of the carbonyl measurement for high, medium, and low values was 5.8%, 7.7%, and 24.7%, respectively.

Plasma-Free F_2-Isoprostane Content

F_2-isoprostanes are lipid peroxidation products produced by free radical mediated nonenzymatic oxidation of arachidonic acid. Free F_2-isoprostanes esterified to plasma lipids were measured by gas chromatography/negative-ion chemical ionization mass spectrometry as described by Morrow and Roberts (*22*). The precision of the assay was ±6%, with an accuracy of 96%. Data are expressed in pg/mL.

Statistical Analysis

The null hypothesis was that renal transplantation would have no significant impact on circulating levels of biomarkers of inflammation and oxidative stress in ESRD patients. The primary outcome measure was plasma IL-6 concentration, a robust predictor of cardiovascular risk in dialysis patients. The study was designed to have 90% power to detect a 50% decrease between pretransplant and 2-month posttransplant IL-6 levels, using a paired *t* test with a 0.05 two-sided significance level. On the basis of our preliminary data from chronic hemodialysis patients showing an average±standard deviation (SD) IL-6 concentration of 18.2±13.9 pg/mL, we estimated that a sample size of 15 patients would be required to detect a 50% reduction in mean IL-6 levels (i.e. from 18.2 pg/mL to 9.1 pg/mL), assuming a standard deviation of the difference of 10.0 pg/mL (*23*). To allow for a dropout rate of up to 20%, we set the enrollment level at 20 patients.

Analyses of within-group changes of baseline and posttransplant values was performed using repeated-measures analysis of variance for normally distributed data or Friedman's test for non-normally distributed data. Univariate comparisons of continuous variables between the study population and healthy controls were assessed using the Student's *t* test for normally distributed data and the Mann-Whitney *U* test for non-normally distributed data. Comparisons of discrete data were completed by chi-square test or Fisher's exact test. We used analysis of covariance for multivariable analyses between the two study groups. Correlations among the study variables were performed by the Spearman rank-order correlation. Results are expressed as mean±SEM unless otherwise stated. All tests of significance were two sided, and differences were considered statistically significant when the *P* value was less than 0.05. The statistical software SPSS version 11.5 (SPSS Inc., Chicago, IL) was used for all analyses.

RESULTS

Characteristics of Patients and Controls

Transplant recipients were, on average, younger than control subjects (38.3±13.7 vs. 48.2±16 years, $P=0.028$), and had a higher percentage of males (58% vs. 36%). Most of the participants in both groups were white (95% and 92%). The four patients not yet receiving renal-replacement therapy at the time of transplantation had GFR less than 15 mL/min, constituting stage 5 CKD. Four (22%) patients had documented comorbid CVD (3 coronary artery disease, 1 stroke) at the time of renal transplantation, 18 (95%) had preexisting hypertension, 12 (63%) had hyperlipidemia, and 3 (17%) were smokers.

The majority of patients had significant restoration of renal function, with median estimated GFRs of 70 mL/min at 1 week posttransplantation and 67 mL/min at 2 months posttransplantation. At both time points, approximately two thirds (68%) of the patients had estimated GFR greater than 60 mL/min, whereas 89% (1 week) and 95% (2 months) had estimated GFR greater than 30 mL/min. Complications within the first 2 months of transplantation included postoperative electrocardiogram changes in one patient with preexisting CVD necessitating cardiac catheterization (no significant coronary occlusion), mild acute cellular rejection occurring in one recipient at 2 weeks, one recurrence of membranoproliferative glomerulonephritis at 1 month associated with declining renal function, and severe gastroparesis in one diabetic patient necessitating discontinuation of mycophenolate mofetil. No patient required dialysis during the posttransplant course of the study.

After transplantation, expected changes occurred in several clinical and laboratory parameters. Serum levels of urea nitrogen and creatinine and estimated GFR improved significantly in all patients, notable within 1 week after renal transplantation ($P<0.05$ for all, data not shown). We observed initial declines in albumin, prealbumin, and hematocrit levels 1 week postrenal transplantation, but these were not significantly different from baseline levels by 2 months after transplantation ($P>0.05$ for all, data not shown), nor did they correlate with concentrations of inflammatory and oxidative stress biomarkers. Body weight increased after renal transplantation, with an average gain of 2.0 kg within the first 2 months ($P<0.05$).

Biomarkers of Inflammation and Oxidative Stress are Increased in Patients with ESRD

We initially compared the study variables in the 19 ESRD patients scheduled for renal transplantation with those of healthy subjects. As depicted in Table 1, the patients had significantly higher levels of the pro-inflammatory cytokines IL-6 (10.5±2.1 vs. 4.0±0.5 pg/mL, $P<0.001$) and TNF-α (53.8±3.6 vs. 11.5±1.1 pg/mL, $P<0.001$), as well as the acute phase reactant CRP (10.1±2.9 vs. 2.8±0.6 mg/L, $P=0.041$) when compared with those of healthy subjects. Likewise, the levels of both oxidative stress biomarkers were significantly elevated as compared with controls: 0.049±0.005 versus 0.029±0.004 nmol/mg protein, $P=0.014$, for plasma protein

TABLE 1. Biomarkers of inflammation and oxidative stress in healthy subjects and ESRD patients before renal transplantation

	Healthy controls (n=50)	ESRD patients Pretransplant (n=19)	*P* value
IL-1β (pg/mL)	6.91±3.03	6.32±2.46	0.672
IL-6 (pg/mL)	4.02±0.47	10.50±2.10	0.001
IL-8 (pg/mL)	4.58±0.74	4.77±0.65	0.684
IL-10 (pg/mL)	1.17±0.15	1.09±0.09	0.413
TNF-α (pg/mL)	11.54±1.13	53.80±3.61	<0.001
CRP (mg/L)	2.83±0.58	10.12±2.90	0.041
Free F2-IsoPs (pg/mL)	46.75±5.06	73.68±9.48	0.002
Carbonyls (nmol/mg protein)	0.029±0.004	0.049±0.005	0.014

Values are presented are mean±SEM.
ESRD, end-stage renal disease; IL, interleukin; TNF, tumor necrosis factor; CRP, C-reactive protein; F2-IsoPs, F2-isoprostanes.

carbonyl content; and 73.7±9.5 versus 46.8±5.1 pg/mL, $P=0.002$, for plasma free F_2-isoprostanes. These baseline differences in biomarker levels were maintained even when adjusted for sex as well as the presence of CVD and diabetes mellitus ($P<0.05$ for IL-6, TNF-α, CRP, carbonyls, and F_2-isoprostanes). Concentrations of IL-1β, IL-8, and the anti-inflammatory cytokine IL-10 were not statistically different from healthy control values.

We also examined the relationships among the various inflammatory and oxidative stress biomarkers in ESRD patients measured before renal transplantation. Not surprisingly, we observed a significant positive correlation of the acute phase protein CRP with IL-6 ($r_s=0.567$, $P=0.011$) and a positive trend of CRP with TNF-α ($r_s=0.427$, $P=0.068$). Interestingly, CRP was also correlated with the oxidative stress marker free F_2-isoprostanes ($r_s=0.477$, $P=0.039$), demonstrating a positive relationship between inflammation and oxidative stress in the setting of advanced uremia.

Biomarkers of Inflammation and Oxidative Stress Decrease after Renal Transplantation

Uremic patients had substantial improvements in inflammatory and oxidative stress biomarkers after restoration of renal function by transplantation. The concentrations of TNF-α ($P<0.001$), plasma protein carbonyls ($P<0.001$), and free F_2-isoprostanes ($P=0.002$) decreased rapidly, with significant changes notable within the first postoperative week (Figs. 1 and 2). Likewise, IL-6 ($P=0.006$) and CRP ($P<0.001$) concentrations showed significant declines from baseline within 2 months after renal transplantation. The final post-transplant measurements of carbonyl content, free F_2-isoprostanes, IL-6, and TNF-α concentrations in renal-transplant recipients were not statistically different from those of healthy control subjects ($P>0.05$ for all). We did not observe any significant change in IL-10 concentrations posttransplantation.

There was a considerable inverse correlation between IL-6 and renal-function indicators ($r_s=-0.571$, $P=0.011$ for GFR; $r_s=0.655$, $P=0.002$ for blood urea nitrogen; $r_s=0.439$, $P=0.060$ for serum creatinine) at 2 months postrenal transplantation but not for TNF-α or CRP. Plasma protein carbonyl content correlated significantly with blood urea nitrogen ($r_s=0.557$, $P=0.016$), although the negative correlation with GFR did not achieve statistical significance. We found no apparent association between F_2-isoprostanes and the level of renal function. Of the biomarkers, only F_2-isoprostanes correlated significantly with body weight ($r_s=0.568$, $P=0.011$) at 2 months posttransplant.

DISCUSSION

In this study, we hypothesized that restoration of renal function by transplantation would improve uremic inflammation and oxidative stress. Indeed, our results demonstrate a rapid decrease in circulating levels of the pro-inflammatory proteins IL-6, TNF-α, and CRP, as well as two established markers of oxidative stress, plasma protein carbonyl content, and F_2-isoprostanes, after renal transplantation. Within 2 months, these values were similar to those of healthy control subjects. On the basis of these observations, one can speculate that restoration of renal function by transplantation improves the chronic inflammation and increased oxidative stress associated with uremia, which may potentially contribute to the improved survival afforded to ESRD patients by renal transplantation. That "normalization" of these markers after renal function was restored suggests that they are not simply epiphenomena of established vascular disease, but this supports the concept that loss of renal function directly contributes to a hyperinflammatory state.

Although renal transplantation appears to improve biomarkers of inflammation and oxidative stress over the short term, data on the effects of dialysis, the most common renal replacement therapy for ESRD, have been inconsistent. Several studies indicate that the dialysis procedure itself incites repetitive inflammation and may thereby promote increased oxidant production (*24*, *25*). In a recent study by our group investigating the impact of initiation of maintenance hemodialysis on inflammation and oxidative stress, we found no significant change in circulating levels of CRP, IL-6, or plasma protein carbonyl content after 12 months of dialysis therapy (*26*). This apparent discrepancy between the effects of renal transplantation and maintenance hemodialysis may reflect multiple mechanisms including more effective clearance or tubular metabolism of solutes and renal regeneration

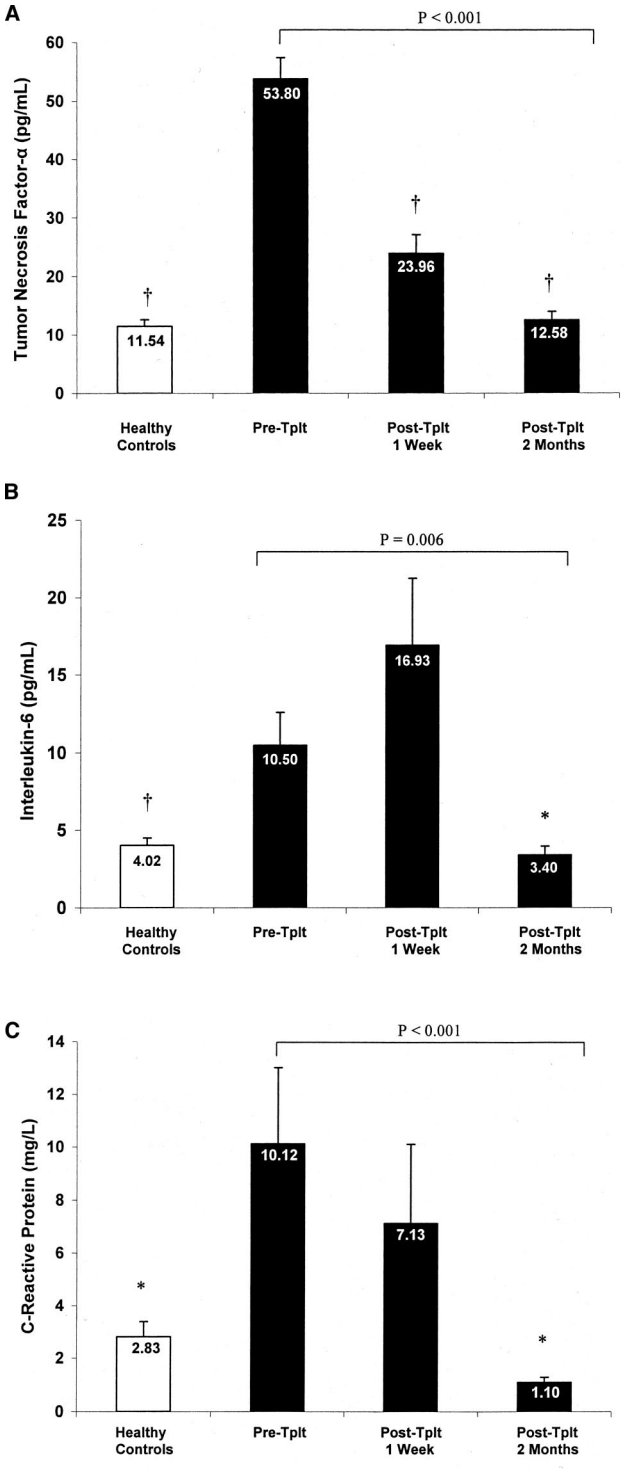

FIGURE 1. Changes in the inflammatory markers tumor necrosis factor-α (A), interleukin-6 (B), and C-reactive protein (C) before and after renal transplantation (Tplt). Bars and error bars represent means±SEM for each time-point. *$P<0.05$ compared with pretransplant values; †$P<0.001$ compared with pretransplant values.

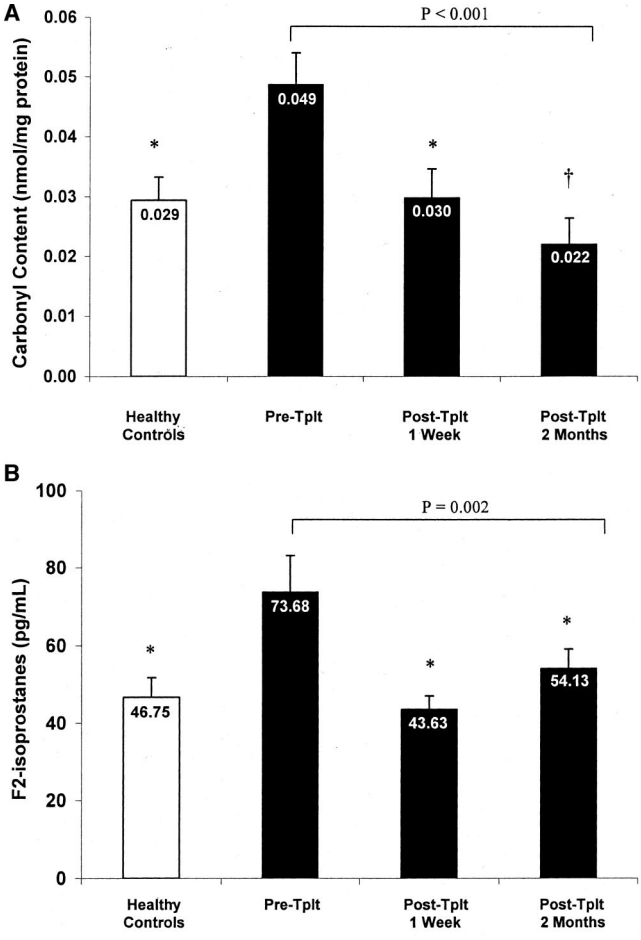

FIGURE 2. Changes in the oxidative stress markers protein carbonyl content (A) and free F-2 isoprostanes (B) before and after renal transplantation. *$P<0.05$ compared with pretransplant values; †$P<0.001$ compared with pretransplant values.

of anti-oxidants by the functioning kidney. Studies show that renal tubular epithelia modify the redox status of ultrafiltrate and resorbate by way of alterations in selective transport and metabolic functions. Specifically, renal tubular function is important in detoxifying reactive aldehydes (carbonyls) (*27*, *28*). The enzyme aldehyde dehydrogenase is highly active in the renal cortex, and loss of this important renal detoxifying mechanism is likely a proximate cause of reactive aldehyde (carbonyl) accumulation in uremia.

Despite improved kidney function and lower inflammatory and oxidative stress biomarkers, the cardiovascular mortality for renal-transplant recipients remains considerably higher than the general population. However, they have a substantial survival advantage over dialysis patients awaiting transplantation, with up to 66% decrease in the long-term risk of death (*15*). For renal-transplant recipients, modulation of nontraditional cardiovascular risk factors such as inflammation and oxidative stress may in part explain the improved survival afforded to them by kidney transplantation.

Although the results presented in this study are intriguing, some potential limitations should be considered. Immunosuppressive medications are necessary to prevent allograft rejection, and it is possible that these agents may attenuate inflammation and oxidative stress. Whether immunosuppressant drugs have a direct effect on markers of oxidative

stress is unknown. This study only examined living-donor transplants with prompt allograft function. Additional studies in deceased donor-transplant recipients with varying levels of early graft function may help to differentiate biomarker changes that result from enhanced renal function. These studies are currently underway in our laboratory. Longitudinal studies, controlled for the different types of immunosuppressive agents and levels of graft function, are also needed to further explore these outcomes.

In summary, chronic uremia is a micro-inflammatory state associated with increased oxidative stress. Although current modalities used in the treatment of CKD have not proven effective in managing these processes, our findings indicate that they are improved upon restoration of renal function by transplantation. We conclude that inflammation and oxidative stress markers are potentially modifiable cardiovascular risk factors in ESRD patients and may represent important targets for therapeutic intervention. The development of such therapies could potentially reduce cardiovascular morbidity and mortality in a number of uremic patients, including those not eligible for transplantation.

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