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ORIGINAL ARTICLE

Correlation between oxidative stress and immunosuppressive therapy in renal transplant recipients with an uneventful postoperative course and stable renal function

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Abstract
Background Reactive oxygen species (ROS) are important mediators of cellular damage and lipid peroxidation is the most important expression of ROS-induced oxidative stress. Recent studies have suggested that increased plasma malondialdehyde (MDA) levels are a consequence of specific immunosuppressive therapies. This study aims at investigating the relation between oxidative stress and immunosuppressive therapies in renal transplant patients with stable renal function and uneventful postoperative course.
Methods The study group included 26 renal patients. Two groups of renal transplant recipients, treated with a different combination of immunosuppressive agents were studied (Group A: CyA, MMF, Steroids and Basiliximab, Group B: Tacrolimus, MMF, Steroids and Daclizumab). All patients had an uneventful postoperative course. Plasma MDA levels were measured before transplantation, 1 and 6 months after. Plasma concentration of endogenous creatinine (Cr) was used as a measure of stable renal function.
Results Levels of MDA were increased before the transplantation in all renal patients (MDA: 7.81 ± 4.81, normal levels: 2.23–4.08 nmol/ml, $P < 0.05$). Combined therapy with CyA was associated with high values of MDA at 6 months measurement after transplantation. However this tendency of increased MDA levels did not achieve a statistical significance (Group A: 6.97 vs. 9.06 nmol/ml, $P>0.05$). On the contrary, statistically significant diminution of MDA levels was observed in Group B patients (Tacrolimus–MMF–steroids) at 6 months measurement after transplantation. (Group B: 8.61 vs. 4.11 nmol/ml, $P<0.02<0.05$).
Conclusions Immunosuppressive combined therapy with CyA was associated with the high values of MDA that were measured posttransplantly. Our study provides strong evidence that Tacrolimus is significantly associated with improved free radical metabolism.

Keywords Renal transplantation · Oxidative stress · MDA · Immunosuppressive · Tacrolimus · Cyclosporine · Reactive oxygen species

Abbreviation
ROS Reactive oxygen species

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Introduction

It is well known that reactive oxygen species (ROS) are important mediators of cellular damage and lipid peroxidation is the most important expression of ROS-induced oxidative stress. The injurious role of ROS in transplanted organs has been demonstrated experimentally and clinically in kidney transplantation [1, 2].

A lot of studies have documented production of free oxygen radicals, generated in the very early period of reperfusion during human kidney transplantation [3, 4]. Cellular sources of oxygen free radicals include the electron transport chain, the microsomal electron transport chain, oxidant enzymes (xanthine oxidase, cyclo-oxygenase), phagocytes, and cellular auto-oxidation of Fe^{2+} and epinephrine. Oxygen radicals cause lipid peroxidation of cell and organelle membranes, disrupting the structural integrity and capacity for cell transport and energy production [5]. Recent reports have suggested that increased plasma malondialdehyde (MDA) levels are a consequence of immunosuppressive therapy [6–8]**.** The Cyclosporine A therapy has been implicated as a putative factor of increased renal lipid peroxidation. On the other hand enhancement of oxidative stress could be owing to an immunologic response to the kidney graft. Oxidative stress could vary with time, expressing potential changes induced by any of the causes of graft dysfunction [9].

The purpose of this study was to investigate the oxidative stress levels in renal transplant recipients with stable renal function, in relation to their immunosuppressive treatment.

Methods

The clinical cohort consisted of 26 renal recipients who underwent renal transplantation between 2000 and 2002. The underlying kidney diseases are shown in Table 1. The study population included two randomized groups of patients, receiving a different combination of immunosuppressive agents (Table 2**).** Recipients of the first group (Group A, *n*=13) were treated with combination of CyA (initial dose 3 mg/kg), MMF (1.5 or 2 g/24 h), Methyl-Prednisolone in progressively diminished dosages and in addition two doses of Basiliximab

Table 1 Causes of renal insufficiency

Diseases	Number of patients (%)
Unknown causes	7 (26.9)
Adult polycystic kidney disease	4 (15.3)
IgA nephropathy	3 (11.5)
Focal segmental glomerulosclerosis	5 (19.2)
Obstructive nephropathy	1 (3.8)
Syndrome Alport	1 (3.8)
Vesico ureteral reflux	2 (7.6)
Malignant hypertension	2 (7.6)
Nephrolithiasis – chronic pyelonephritis	1 (3.8)

Plasma MDA (nmol equivalents/ml) and Cr levels. Values are means ± SD

(The 1st and the 4th postoperative day). The expected blood level of CyA was 900 mg/ml, 2 h following the drug's uptake. Immunosuppression of the second group (Group B, *n*=13) consisted of MMF (1.5 or 2 g/24 h), Tacrolimus (0.05 or 0.1 mg/24 h), Methyl-Prednisolone in progressively diminishing doses, Daclizumab (Five doses postoperatively). The study protocol recommended expected blood levels of Tacrolimus 5 mg/ml. All patients started Methyl-Prednisolone on the first postoperative day in a dose of 20 mg/day and subsequently the dose was tapered to reach a dose of 16 mg/day by 1st month, 8 mg/day by 3 months and 4 mg/day maintenance dose by 6 months. All patients were followed-up with clinical and laboratory examination, and by dimercaptosuccinic acid (DMSA) scanning.

All patients in our study had an uneventful postoperative course, stable renal function (Cr < 1.8 mg/dl) and did not present findings that implied rejection. All patients received living related grafts. Plasma MDA levels were measured before and 1 and 6 months following transplantation. The samples were obtained immediately before haemodialysis, and were preserved in tubes with anticoagulant and antioxidative agent (BHT 0.2% in methanol), to measure 2-thiobarbituric acid reactive substances (TBARS). Plasma was separated by low-speed centrifugation (3500 g/min for 15 min). A 0.5 ml aliquot of each sample was added to a tube containing 3 ml of 0.05 N HCl and mixed. In each tube, 1 ml of 46 mmol/l 2-thiobarbituric acid (TBA) was added. The tubes were boiled for 30 min and allowed to cool. These mixtures were then added to tubes containing 4 ml

Table 2 Clinical and demographic characteristics of the study groups

	Group A (n=13)	Group B (n=13)
Immunosuppressive medication	MMF, CyA, steroids	MMF, Tacrolimus, steroids
Gender (male/female)	9/4	11/2
Age (years)	37.78±11.58	37.0±9.2
Body mass index (kg/m^2)	25.1±3.3	23.2±2.5
Plasma creat. 1 month (mg/dl)	1.4±0.27	1.5±0.75
Plasma creat. 6 months (mg/dl)	1.31±0.32	1.45±0.27
(MDA) pretransplantly	7.06±2.48	8.61±3.2
(MDA) posttransplantly 1 month	6.97±2.39	4.55±1.47
(MDA) Posttransplantly 6 months	9.06±2.53	4.11±2.19
Coexistent diseases relating to cardiovascular status		
Hyperlipidemia	4/13	2/13
Heart coronary disease	1/13	0/13
Malignant hypertension	1/13	1/13
Hypertension	1/13	2/13
Active smokers	2/13	2/13

methanol/butanol 3:17 and mixed. After centrifugation at 2500 g/min for 20 min, 1.5 ml of each supernatant was taken and the absorbance at 535 nm was measured. The TBARS were calculated as MDA equivalents, using freshly diluted malondialdehyde bis (1,1,3,3-tetraethoxypropane) as the standard. Malondialdehyde was prepared by hydrolysis of 1,1,3,3-tetraethoxypropane with concentrated H_2SO_4. Plasma concentration of endogenous creatinine (Cr) was used as the measure of stable renal function.

Data was expressed as means ± SD. A statistic analysis of our results was performed utilizing ANOVA and Robust tests of Equality of Means (Welch, Brown–Forsythe). Also non-parametric Wilcoxon Signed Ranks test was used for paired samples comparison and Pearson's correlation coefficient was computed in order to examine linear dependencies. Differences were considered to be significant at the level of 5% (P<0.05).

Results

Levels of MDA were increased before the transplantation in all renal patients. (MDA: 7.81 ± 4.81, normal levels: 2.23–4.08 nmol/ml, P<0.05) (Table 2, Fig. 1). In our study immunosuppressive combined therapy with CyA at 6 months after transplantation was associated with high values of MDA. However this tendency of increased MDA levels did not achieve statistical significance (Group A: 6.97 vs.

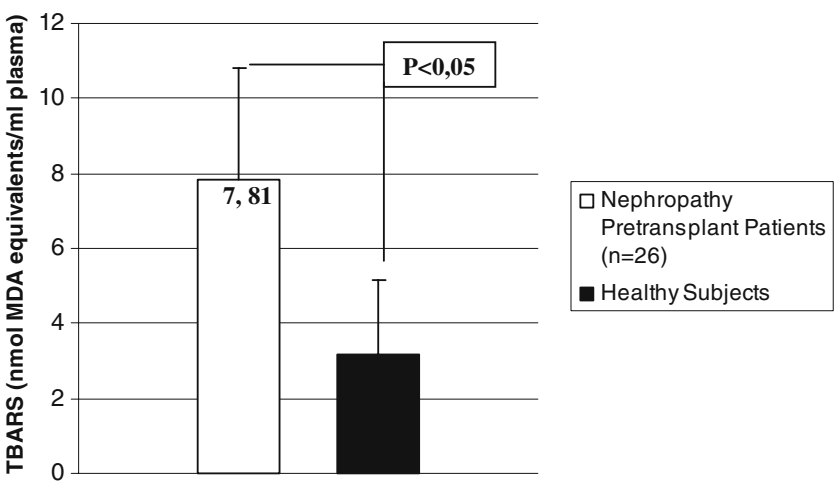

Fig. 1 Plasma TBARS values in nephropathy pretransplant patients, compared to normal levels in healthy subjects

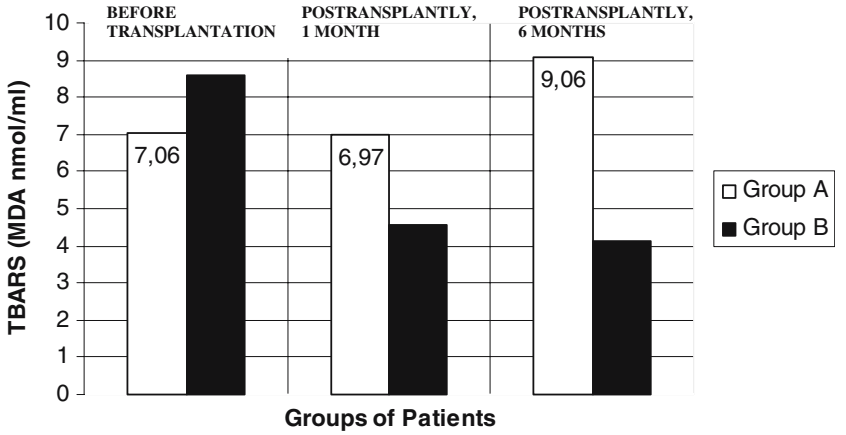

Fig. 2 Correlation of immunosuppressive therapy with oxidative susceptibility (TBARS were calculated as malondialdehyde (MDA) equivalents in plasma samples)

9.06 nmol/ml, $P>0.05$). On the contrary, a statistically significant decrement of MDA levels was observed in Group B patients (Tacrolimus–MMF–steroids) at the 6-month period (Group B: 8.61 vs. 4.11 nmol/ml, $P<0.02<0.05$) (Fig. 2).

The results that occurred by performing a direct comparison between the two groups (ANOVA—Robust tests of Equality of Means) were consistent and came to confirm the uniformity of the two groups pretransplantly and the significant differences in 1 and 6 months (Pretransplantly: 7.06 vs. 8.61 $P=0.46>0.05$, 1 month: 6.97 vs. 4.55 $P=0.04<0.05$, 6 months: 9.06 vs. 4.11 $P=0.006<<0.05$) A detailed analysis showed that changes of MDA values in the immunosuppressive groups were not correlated with sex or age. No difference in Cr levels between the two groups was found.

Discussion

Uncertainty exists concerning the determination of oxidative status of a transplanted patient and additively for the interpretation of the potential variability. Several factors have been implicated with induction of oxidative stress in transplant patients. Free radical production, leading to lipid peroxidation phenomena, can occur within the early phase of kidney revascularization as a consequence of the critical period of parenchymatic ischemia [10, 11]. Chronic rejection (CR), the most important cause of long-term graft failure, is associated with enhancement of oxidative stress [12]. This observation suggests that oxidative stress may participate in the development and progression of vascular lesions [13]. Therefore, the time when samples are obtained in the postoperative period, may be a relevant pathophysiological factor associated with graft function and oxidative stress expression. Also the immunosuppressive therapy has been implicated as a putative factor of increased renal lipid peroxidation. The CyA treatment has been associated with stimulation of oxygen radical formation in the kidney [7].

According to the results of our study, levels of MDA were increased before the transplantation in renal patients (MDA: 7.81 ± 4.81 nmol/ml, Normal levels: 2.23–4.08 nmol/ml, $P<0.05$). This observation has double interest. First, it confirms reported studies that suggest an imbalance between oxidants and antioxidants agents in uremic patients. Disturbances in antioxidant systems occur in the early stages of chronic uremia and gradually increase with the degree of renal failure [14]. Also oxidative stress is further exacerbated by haemodialysis, as evidenced by increased lipid peroxidation and low antioxidant levels in dialysis patients [15]. Second, it emphasizes the fact that renal patients already have an increased free radical production and accessional factors related to oxidative stress, could alter additively the balance between oxidants and antioxidant defences.

In our study immunosuppressive combined therapy with CyA–MMF, at 6 months after transplantation, was associated with high values of MDA, even if this tendency of increased levels was not found statistically significant. (Group A: 6.97 vs. 9.06 nmol/ml, $P>0.05$). A statistically significant diminution of MDA levels was observed in Tacrolimus–MMF treated patients at the 6th month

measurement, compared to the preoperative levels (8.61 vs. 4.11 nmol/ml, $P<0.02$) and also compared to respective MDA levels of Group A patients (1 month: 6.97 vs. 4.55 $P=0.04$, 6 months: 9.06 vs. 4.11 $P=0.006$). Based on these findings, we suggest that cyclosporine A may be associated with a tendency to induce free radical production during the posttransplant period. This consideration does not exclude an increased oxidative stress in long-term CyA treatment but needs further investigation for its support.

The present study evaluates the different effects of diverse immunosuppressive agents in oxidative stress expression after renal transplantation. Only transplant recipients with uneventful postoperative course and stable renal function were included, in order to investigate comparatively the role of different immunosuppressive agents and to avoid increased oxidative stress due to graft dysfunction. The design of the study was simple but straightforward in order to investigate and possibly correlate graft survival after renal transplantation with variability of oxidative stress. There are certain points that must be clarified:

(a) The populations of the two groups were relatively small. However the random selection procedures that were used to create the two study groups assured the uniformity of the groups quantitatively and qualitatively, enhancing the accuracy of the statistical analysis.

(b) The injurious role of ROS has been demonstrated experimentally and clinically in kidney transplantation. Potential variability of oxidative stress may be sensitive but not a specific factor for the graft's function for two reasons. First, several factors may be involved in induction of oxidative stress in transplant patients. So, the clinical status of these patients should be strictly determined in order to differentiate the possible variability in oxidative stress. Second, increased oxidative status may be correlated with a clinical effect in the short or long-term period, depending on the gravity of the implicated disorder. In fact, in our study at the 6 month follow-up period there was no difference in levels of Cr between the two different groups.

(c) Enhancement of oxidative stress can not be considered a prognostic factor for the graft's outcome but can reflect a tendency for increase of graft's cellular ''entropy'' and possible future damage.

In conclusion, an increased oxidative stress in pretransplant patients suggests an imbalance between toxic oxidants and antioxidants defences. Our study provides strong evidence that Tacrolimus is significantly associated with improved free radical metabolism.

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