CARDIOVASCULAR RISK FACTORS AFTER RENAL TRANSPLANTATION

THE IMPACT OF TREATMENT WITH CYCLOSPORINE A

Cardiovascular risk factors after renal transplantation. The impact of treatment with cyclosporine A / Marinus Adriaan van den Dorpel
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Cardiovasculaire risicofactoren na niertransplantatie;
de invloed van behandeling met cyclosporine A

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CARDIOVASCULAR DISEASE AFTER RENAL TRANSPLANTATION

Introduction

Renal transplantation is presently the renal replacement therapy of choice for most patients suffering from end-stage renal failure, mainly because of the gain in quality of life after successful allografting. During the past decade the results of renal transplantation, in terms of incidence of rejection episodes and graft loss because of acute rejection, have improved substantially due to the introduction of cyclosporine A. However, it appears that, despite the decrease in morbidity rates following renal transplantation, in the long run the improvement in patient survival is rather limited (1).The success in terms of graft survival without a parallel improvement in patient survival, also emphasizes that comorbid conditions such as cardiovascular disease, become increasingly important for the long-term prospects of this patient group.

The morbidity and mortality from atherosclerotic cardiovascular disease in patients with end-stage renal failure is far in excess of that in the normal population. Data from the Registry of the European Dialysis and Transplant Association (EDTA) (2) show that patients receiving renal replacement therapy have a 15- to 20-fold increased risk of myocardial ischemia and infarction when compared with age- and sex-matched healthy control subjects. Especially younger and diabetic patients appear to be at risk compared to controls. It is estimated that cardiovascular disease accounts for 40-60% of deaths among patients who have lost their native renal function. Although the cardiovascular death rate in renal transplant patients is approximately half that of the total population with renal failure (which is mainly due to patient selection), it remains much higher than that of the normal population when matched for demographic variables. In one study, the incidence of atherosclerotic complications was found to be closely linked to pretransplant evidence of such disease (3). However, the incidence was also increased in those patients who had no evidence of atherosclerosis before renal transplantation (4).

The data from the European Registry are confirmed by the outcome of an analysis of the causes of graft loss after renal transplantation, performed on data from Australia and New Zealand (5), showing that death of the recipient with a functioning graft accounted for approximately two-thirds (67%) of all graft losses between 1 and 5 years after transplantation and one-third of graft loss after 5 years. Reports from the EDTA have

repeatedly emphasized that cardiovascular disease is the cause of death in about one third of renal transplant patients, compared with about 22% due to infectious causes (2). Follow-up analyses have also shown that the death rate from cardiovascular causes did not decrease over the years, in contrast to mortality from infection, which halved between 1982 and 1987 (2). These observations highlight the already increased cardiovascular risk of renal transplant recipients from the onset and suggest an accelerated progression of the atherosclerotic disease process after transplantation.

The nature of the association between renal failure and cardiovascular disease is still unclear. Attempts to understand the causes of this particularly high rate of cardiovascular complications in renal failure and renal transplant recipients require a thorough appreciation of the risk factors known to contribute to cardiovascular disease in the general population, such as hyperlipidemia, glucose intolerance, smoking and, in particular, hypertension. Besides these well-known risk factors, disturbed diurnal blood pressure regulation, impaired fibrinolysis as a manifestation of endothelial dysfunction, increased oxidant stress and raised plasma homocysteine levels are newly identified contributors to the development of atherosclerotic cardiovascular disease in the general population, which will be briefly reviewed in the following sections.

Twenty-four hour blood pressure

Hypertension is one of the established risk factors for cardiovascular disease. It is well known that almost all patients with renal disease become hypertensive as they progress towards end-stage renal failure. In this patient group, control of salt and fluid balance is the cornerstone of adequate blood pressure regulation. Despite restoration of renal function, hypertension is a very common complication after transplantation, occurring in up to 60% of graft recipients (6).

The impact of different immunosuppressive regimens on blood pressure has been confirmed by studies showing that conversion from cyclosporine A (CsA) to azathioprine (AZA) and also withdrawal of corticosteroids reduces the incidence of hypertension by about 50% (7,8). In non-transplant patients, even low doses of cyclosporine elevate blood pressure in the majority of patients (9). The mechanism of cyclosporine induced hypertension is much debated, with some workers suggesting that it is renally mediated and others that it is a separate entity from cyclosporine nephrotoxicity (10-12). CsA causes afferent renal vasoconstriction, leading to inappropriate sodium reabsorption,

expansion of the extracellular volume and fluid retention due to a distal tubular effect (6, 10, 11). In addition, the systemic effects of CsA, such as enhanced sympathetic tone, interference with vasoactive factors such as endothelin-1, prostanoids and nitric oxide, or elevation of intracellular calcium levels contribute to the development of hypertension during CsA treatment (reviewed in 11 and 12).

Although no prospective data are available, it is very likely that hypertension after renal transplantation is as important, or even more so, as a risk factor for cardiovascular disease than in the general population. In a retrospective study, the presence of hypertension was associated with both ischemic heart disease and cerebrovascular disease (1). In clinical practice, adequate blood pressure control after renal transplantation is difficult to achieve. A recent EDTA survey showed that in about one-third of renal graft recipients, systolic and diastolic blood pressure was greater than 150 and 90 mmHg respectively, despite the use of antihypertensive therapy (2).

Until now, antihypertensive treatment has been guided by infrequent clinic or office blood pressure measurements. Taking into account that blood pressure varies considerably between individuals and during the day, random measurements clinic blood pressure may not be representative of the blood pressure at other times. Studies in patients with essential hypertension have shown that the total hypertensive load, represented by the time-averaged blood pressure, is a better risk indicator for cardiovascular disease than relatively small numbers of blood pressure recordings in the clinic (13,14). In renal transplant patients ambulatory blood pressure was closely correlated to the severity of echocardiographic left ventricular hypertrophy, which is at present the most sensitive measure of target organ damage (15).

The technique of ambulatory blood pressure monitoring has been further developed and has rapidly expanded as both an instrument for research purposes and in clinical practice. Consensus has been reached on a large number of technical issues, which will not be discussed in detail, but have been published before (15,16). The technique of non-invasive blood pressure recording has been compared with intra-arterial measurements, showing a close similarity between 24-hour averages of both methods, even when the measurement intervals were as long as 1 hour. In general, ambulatory recording does not elevate nighttime blood pressure and is a useful method to assess diurnal blood pressure variability. There is also evidence that group means of blood pressure level and of diurnal blood pressure profile can be accurately reproduced with repeated measurements in the

same subjects. In addition, the within-subject reproducibility of ambulatory measurements is better than that of conventional blood pressure measurements (15). This enables researchers to draw meaningful conclusions from studies with a relatively small number of participants and gives clinicians a more reliable parameter of their therapeutic strategy.

In patients with advanced renal failure or on intermittent hemodialysis and in renal transplant recipients, diurnal blood pressure variation is blunted (17,18). Sodium retention with concomitant volume expansion appears to be a major determinant in the pathogenesis of this phenomenon, although in patients with Cushing's disease with nocturnal hypertension, glucocorticoid, but not mineralocorticoid excess was associated with impairment of the night-time fall in blood pressure (19).

Endothelial vasodilator dysfunction

The endothelium plays a crucial role in the vessel wall because, apart from serving as the barrier between the blood and extravascular space, it releases a large variety of mediators involved in the regulation of vascular tone, hemostasis and cell-cell interaction (20). Endothelial cells alter vascular tone by shifting the balance between vasodilator substances, such as prostaglandins and endothelium-derived relaxing factor (identified as nitric oxide) and vasoconstrictor substances, including endothelin-1 and angiotensin II. Nitric oxide (NO) is formed from the amino acid L-arginine by the enzyme NO-synthase and modulates the development of atherosclerosis at different levels (21). NO inhibits platelet aggregation and adhesion, suppresses the growth of smooth muscle cells, impairs the release of endothelin-1 and also decreases the binding of leukocytes and monocytes to the endothelial luminal surface (reviewed in 20). Because all these events all are thought to be involved in the etiology of atherosclerosis, NO may be a potent anti-atherosclerotic compound in addition to its vasodilator actions. Until recently, the loss of the endothelial cell layer itself, rather than endothelial dysfunction was regarded an important step in the pathogenesis of atherosclerosis. Alternatively, atherosclerosis is increasingly seen as an inflammatory process, opposed to an accumulation of lipids in the vessel wall (22). Endothelial cells have the capacity to recruit inflammatory cells and modulate their function via the release of NO. NO appears to inhibit the activation of transcription factors, like nuclear factor- κB, that mediate the transcription of genes involved in the development of atherosclerosis. NO mediates the vasodilatation induced

by various substances such as bradykinin and acetylcholine and also modulates the vasoconstriction caused by other factors such as angiotensin II and endothelin-1 (20,23).

In vivo endothelial vasodilator function in humans can be assessed in various ways. The forearm model is often used to examine the response of the endothelium on intra-arterial administration of compounds with predominantly endothelium-dependent or endothelium-independent vasodilator actions. Venous occlusion plethysmography is a validated method for measurement of changes in forearm blood flow during pharmacological studies (24,25). The venous return from the arm is obstructed, while arterial blood flow continues unimpeded, resulting in an increase in forearm swelling at a rate proportional to the rate of arterial blood flow. The rate of forearm swelling can be calculated from the change in forearm circumference, which is measured by means of a strain gauge placed around the forearm. With the use of an automated computer-based system, on-line measurements of the response of the forearm vascular bed during pharmacological interventions are available. The results are expressed as flow per unit forearm volume (usually milliliters per 100 ml forearm volume), or the forearm resistance can be calculated as perfusion pressure divided by forearm blood flow (usually expressed as arbitrary units). In patients with several diseases, such as essential hypertension, diabetes mellitus and hypercholesterolemia, functional impairment of endothelial vasodilator function has been found with this technique (26-28). Until now, no studies have been published about systemic endothelial vasodilator function in transplant recipients.

Like in the systemic vasculature, production of NO by endothelial cells also participates in the regulation of vascular tone in the kidney (29). This is illustrated by the marked renal hyperperfusion which is caused by infusion into the renal artery of substances such as acetylcholine or bradykinin, which are known to enhance NO production. One of the most widely used methods to increase renal blood flow and glomerular filtration rate is the oral or intravenous administration of protein or an amino acid solution (30). Further research has demonstrated that this effect is also caused by the enhancement of intrarenal NO production, although other mechanisms appear to be important as well (31). This contention is corroborated by observations that the NO-precursor L-arginine is a potent renal vasodilator and that co-adminstration of the NO-synthase inhibitor L-NAME abolishes the renal hyperperfusion caused by L-arginine (32).

Therefore, the renal response to amino acid infusion and especially L-arginine, can be used to assess the integrity of endothelial vasodilator function.

Fibrinolysis

Thrombosis of coronary arteries is now generally recognized as the precipitating event in the transition from stable to acute coronary artery disease, i.e. unstable angina, acute myocardial infarction and sudden cardiac death (33). Besides local stimuli such as disruption of plaques, systemic pro-thrombotic factors may add to thrombus formation and subsequently to the onset of overt cardiovascular disease. Endothelial dysfunction, but also other factors such as tissue hypoperfusion and elevated concentrations of procoagulant proteins, e.g. fibrinogen and factor VII, may increase the thrombosis risk (34-36). Impaired endothelial function, may result in impaired fibrinolysis, besides local vasoregulatory disturbances.

Data to improve our understanding of the role of hemostatic factors in atherosclerosis and coronary artery disease are derived from large epidemiological surveys and cross-sectional studies (37). Recently, however, the knowledge of this association has expanded considerably and is added to new insights into the mechanisms that regulate the plasma levels and activity of hemostatic proteins.

The reports from the Northwick Park Heart Study demonstrated a strong association between baseline plasma fibrinogen concentration and Factor VII activity with the risk of subsequent coronary events. Later, a close relation between fibrinolytic activity and the risk for future coronary artery disease was found in the same study (38). This suggests that low fibrinolytic activity is a strong determinant of premature coronary artery disease.

In apparent contrast to this finding, higher and not lower concentrations of tissue plasminogen activator (tPA) antigen were found in participants of the Physician's Health Study, who later developed myocardial infarction (39). However, the elevated tPA may only reflect the higher concentration of circulating complexes of tPA and its natural inhibitor plasminogen activator inhibitor-1 (PAI-1). It is therefore likely that the association between a high tPA concentration and coronary artery disease is explained by high PAI-1 activity and reduced rather than enhanced fibrinolytic activity. Longitudinal studies have also provided evidence that impaired fibrinolysis is implicated in the pathogenesis of atherosclerotic disease (36).

As mentioned earlier, the incidence of cardiovascular disease after renal transplantation is high and, like in the normal population, this is possibly related to disturbance of the fibrinolytic system. After renal transplantation fibrinolytic activity is impaired, especially in the subgroup of patients treated with CsA (40,41). This may be caused by the elevation of plasma triglycerides during CsA. In diabetic and obese subjects, hypertriglyceridemia is associated with a reduction in fibrinolytic activity (41). The impairment of fibrinolysis during CsA treatment can be ameliorated by administration of fish-oil, suggesting that alterations in prostanoid metabolism are involved in CsA-induced hypofibrinolysis (42). CsA treatment is also accompanied by a high incidence of non coronary vascular events such as venous thrombosis and pulmonary embolism (41). In addition, CsA nephrotoxicity is characterized histologically by fibrin and platelet deposition in renal arterioles, presumably also due to the prothrombotic effects of CsA (43).

Lipid peroxidation

Hypercholesterolemia is generally recognized as a primary risk factor for atherosclerosis. In renal transplant recipients plasma total cholesterol is increased, mainly due to an elevated cholesterol content of low-density lipoprotein (LDL) particles (44,45). In addition, cholesterol and triglyceride concentrations in very low density lipoprotein particles are also increased (46). High density lipoprotein cholesterol (HDL) levels do not appear to differ much from those of the general population (44). Besides the negative influence of quantitative differences in plasma lipoprotein classes, qualitative differences may be equally important for the cardiovascular risk profile of renal transplant recipients.

Plasma low-density lipoproteins are the principal source of lipids found in atherosclerotic lesions. Accumulation of LDL in the vessel wall intima is one of the earliest events in the development of atherosclerosis (reviewed in 47). LDL is taken up by the LDL receptor, present on vascular endothelial cells and macrophages. This mechanism plays a crucial role in the regulation of plasma LDL levels, as is apparent in patients with familial hypercholesterolemia. However, most of the LDL present in the arterial wall is not taken up by mechanisms that are mediated by the classic LDL-receptor, which is down-regulated by the intracellular accumulation of LDL. An additional so called "scavenger or acetylated LDL" receptor has been identified, which is also present on endothelial cells, Kupffer cells and macrophages. The uptake of acetylated or oxidized LDL via this receptor exceeds the uptake of unmodified LDL via the LDL receptor and appears not to be

controlled by intracellular LDL. This scavenger receptor recognizes derivatized amino groups of lysine residues of proteins such as apolipoprotein B. These lysine derivatizations influence LDL metabolism, and also profoundly affect the biological activity of lipoproteins.

The occurrence of oxidation of LDL *in vivo* has been demonstrated by several lines of evidence (48,49). The first consists of the immunocytochemical demonstration of oxidized LDL (OxLDL) in atherosclerotic lesions. Although immunocytochemistry has documented the presence of several epitopes that are typical of oxidative modification within lesions, these epitopes need not exclusively reflect the presence of OxLDL, but may also be present on other (lipo)proteins. The second observation that favors *in vivo* LDL oxidation is the demonstration that LDL which is extracted from atheroma lesions contains oxidation-specific epitopes and shares chemical and biological activities with *in vitro* oxidized LDL. This *in vivo* oxidized LDL is also recognized by macrophage scavenger receptors. The presence of circulating antibodies directed against OxLDL provides the third line of evidence. Human serum contains IgM and IgG antibodies that predominantly recognize the malondialdehyde-lysine epitope. It has been established that OxLDL is highly immunogenic and could therefore induce antibody formation *in vivo*. The correlation between antibody titer and the severity or rate of progression of atherosclerosis is not precisely known, although some studies have found that antibody titers were higher in subjects with progressive atherosclerosis. It is unknown whether circulating anti OxLDL antibodies are capable of penetrating the arterial wall, bind to OxLDL and evoke a low grade inflammatory reaction which is often present in atherosclerotic lesions. Finally, the presence of immune complexes between OxLDL and autoantibodies in atherosclerotic lesions also supports the contention that LDL is oxidized *in vivo*. The rapid uptake of OxLDL by macrophages is only one way by which oxidation may contribute to the formation of atherosclerotic lesions. Recent *in vitro* and *in vivo* studies indicate that OxLDL exerts numerous events that may participate in atherogenesis.

The complex mechanisms for LDL oxidation *in vivo* are not fully elucidated, as it is not known whether any of the systems identified *in vitro*, stimulate LDL oxidation *in vivo*. Metal ions, especially Fe and Cu, either free or protein bound, appear to be involved in the formation of oxidized lipids and the development of atherosclerotic lesions. However, increased LDL oxidation and premature atherosclerosis is not a feature of hemochromatosis or Wilson's disease. Other studies suggest that the generation of

reactive oxygen intermediates by smooth muscle cells and macrophages might promote LDL oxidation *in vivo*.

Renal failure is associated with increased susceptibility of LDL particles to oxidation *in vitro* and *in vivo* (50), due to either accumulation of oxidative compounds or deficiency of anti-oxidants. There are few studies on the impact of restoration of renal function by transplantation on parameters of lipid oxidation.

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Chapter 2

AIM OF THE STUDIES

Considering the ample evidence indicating that cardiovascular disease is the most common cause of morbidity and mortality after renal transplantation, the question can be raised whether the use of immunosuppressants such as CsA, may have a negative impact on factors that determine long-term prognosis, with emphasis on the chance to develop overt cardiovascular disease. In order to assess the potentially negative influence of CsA on recently discovered risk factors for cardiovascular disease in renal transplant patients, we designed a study protocol which enabled us to analyze the changes that occurred in several risk factors for cardiovascular disease.

On the outpatient clinic of the Department of Internal Medicine I, we performed a randomized prospective study of the short-term and long-term effect of conversion of the standard immunosuppressive treatment, based on CsA, to a regimen without CsA, based on AZA. For this trial, all renal transplant recipients who were at least 6 month after renal allografting, were eligible. A subset of non-diabetic patients, without pretransplant hypertension and with a stable serum creatinine below 200 μmol/l, participated in the studies described in this thesis.

The aim of these studies was:

1. To assess the impact of conversion from CsA to AZA on the regulation of diurnal blood pressure variation
2. To determine whether impaired endothelial vasodilator function plays a role in the pathogenesis of CsA-induced hypertension.
3. To analyze the differences in lipid metabolism and composition between CsA-treated renal transplant recipients and matched healthy control subjects.
4. To analyze the changes in lipid composition, susceptibility of LDL to oxidation and *in vivo* LDL oxidation in renal transplant recipients following withdrawal of CsA.
5. To evaluate the influence of conversion from CsA to AZA on plasma fibrinolytic activity, and correlate changes in parameters of the fibrinolytic system with plasma levels of prostanoids.
6. To investigate renal vasodilator capacity during CsA and AZA, evoked by infusion of L-arginine, the substrate of NO-synthase for the production of NO.

Chapter 3

CYCLOSPORINE A IMPAIRS THE NOCTURNAL BLOOD PRESSURE FALL IN RENAL TRANSPLANT RECIPIENTS

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ABSTRACT

In renal transplant recipients hypertension and a diminished nocturnal blood pressure fall is frequently found. To investigate whether this diminished nocturnal blood pressure fall is related to the use of cyclosporine A or to other factors, such as the use of glucocorticoids, 24-hour ambulatory blood pressure measurements were performed in 18 renal transplant recipients, both before and 16 weeks after conversion from cyclosporine A to azathioprine. Renal blood flow and glomerular filtration rate were estimated from ^{131}I-hippurate and ^{125}I-iothalamate clearances, and plasma concentrations of renin, atrial natriuretic peptide, norepinephrine, prostaglandin E_2 and thromboxane B_2 were determined. During cyclosporine A mean 24-h blood pressure was 117±3 mmHg and the nocturnal fall in blood pressure was 4±9 mmHg. A non-dipping diurnal blood pressure pattern was present in 13 patients. After conversion to azathioprine mean 24-h blood pressure decreased to 109±3 mmHg ($p<0.001$), the nocturnal fall increased to 9±6 mmHg, and the number of patients with a non-dipping diurnal blood pressure pattern decreased to 9. The nocturnal fall in heart rate (17±10 bpm) during cyclosporine A did not change after conversion. Body weight, plasma concentrations of norepinephrine and renin did not change. Plasma concentrations of prostaglandin E_2 and thromboxane B_2 decreased after conversion, as did plasma atrial natriuretic peptide. Renal blood flow and glomerular filtration rate increased after conversion. In conclusion, cyclosporine A appears to be involved in the disturbance of the circadian blood pressure rhythm in renal transplant recipients. Although the precise mechanism is unclear, the elevated plasma atrial natriuretic peptide and slightly suppressed plasma renin concentration suggest that intravascular volume expansion may contribute to the observed hemodynamic alterations.

INTRODUCTION

The normally occurring nocturnal decline in blood pressure has been reported to be absent or attenuated in heart, liver and kidney transplant recipients (1-7). Existing evidence suggests that the use of glucocorticoids is associated with this hemodynamic abnormality.

Van den Borne et al. found that glucocorticoid therapy dose-dependently attenuated or abolished the nocturnal fall in blood pressure in liver transplant recipients (5). Furthermore, the same group of authors also showed that the reappearance of the

normal nocturnal decline in blood pressure in cardiac transplant recipients was related to the reduction of the dose of glucocorticoids after transplantation (8). Moreover, a reduced or absent nocturnal decline in blood pressure has been observed in patients with endogenous or exogenous hypercortisolism (9).

The use of cyclosporin A (CsA) is frequently accompanied by the development of hypertension (4,7). Several causative mechanisms have been postulated, including an increase in sympathetic nerve activity, a decrease in renal function, disturbance of the synthesis and release of prostaglandins and endothelin-1 and sodium retention (10-14). Apart from inducing hypertension, CsA, alone or in combination with glucocorticoids, may contribute to the attenuation of the nocturnal decline in blood pressure after transplantation.

In order to gain more insight in the role of CsA in the attenuation of the nocturnal decline in blood pressure, kidney transplant recipients were studied during a CsA- and an azathioprine (AZA) -based immunosuppressive regimen. In both instances, the dose of glucocorticoids was kept at a constant level.

METHODS

Patients.

Patients were recruited among renal transplant recipients who were enrolled in a prospective randomized clinical trial, which was designated to evaluate the effects of two different immunosuppressive regimens, i.e. prednisone combined with CsA or prednisone combined with AZA, on long-term graft function and incidence of rejection episodes. For this trial CsA-treated renal transplant recipients who were 6 months or longer after transplantation, were randomly allocated to either continuation of CsA treatment, or conversion from CsA- to AZA-based immunosuppression.

For the present study renal transplant recipients without preexistent hypertension, a clinic blood pressure of 150/95 mmHg or higher during antihypertensive treatment and who were allocated to conversion from CsA to AZA, were selected. Patients with diabetes mellitus, previous graft rejection or signs of autonomic neuropathy were excluded. The first 18 consecutive patients fulfilling these criteria and willing to give written informed consent were studied. About 50% of the patients who were converted from CsA to AZA did not meet all criteria, mainly because of preexistent hypertension which could not be attributed to the use of CsA.

Study design

The first study was performed while patients were on CsA and the second study 16 weeks later when patients were on AZA therapy. During both studies the patients used the same dose of prednisone. All antihypertensive medication (betablockers in 13 patients and calciumchannel blockers in 8 patients) was discontinued 3 days prior to the studies. On both study days patients arrived in the cardiovascular research laboratory after an overnight fast. At arrival they were weighted and subsequently a small catheter (Venflon, Viggo Spectramed, Helsingborg, Sweden) was inserted in a forearm vein of each arm for the infusion of radiopharmaceuticals and blood sampling. After the renal function studies, patients were fitted with the equipment for 24 hour ambulatory blood pressure monitoring and were discharged from the hospital. The study protocol was approved by the local Medical Ethics Committee and all procedures were performed in accordance with institutional guidelines.

Measurements

Renal hemodynamics

Effective renal plasma flow (ERPF) and glomerular filtration rate (GFR) were estimated from the urine clearance of ^{131}I-hippuran and ^{125}I-thalamate (Amersham International Plc, Little Chalfont, Buckinghamshire, UK) as previously described (15). The mean of two calculated clearances of ^{131}I-hippurate and ^{125}I-thalamate was used for further analysis. During the renal function studies arterial pressure was measured by an automated oscillometric device (AccuTorr2, Datascope Corp., Montvale, NJ). Renal blood flow (RBF) was calculated by dividing effective renal plasma flow by (1- hematocrit). Renal vascular resistance (RVR) was calculated by dividing mean arterial pressure (MAP) by RBF. Before the start of the renal function studies a blood sample was taken for the determination of CsA 12-hour trough blood levels (CycloTrac SP, IncStar Corp., Stillwater, MN).

Vasoactive hormones

After 30 minutes of supine rest during the renal function studies blood was sampled for measurement of plasma concentrations of norepinephrine, atrial natriuretic peptide (ANP) and renin. Plasma norepinephrine concentration was measured by fluorimetric

detection after high-performance liquid chromatographic separation as described previously (16). Plasma ANP concentration was measured by means of a radioimmunoassay using a commercially available kit (17). Plasma renin concentration was measured by an immunoradiometric assay as previously described (18).

24-Hour blood pressure monitoring

For ambulatory blood pressure monitoring the oscillometric SpaceLabs monitor model 90207 was used (SpaceLabs Inc., Redmond, WA). Blood pressure was measured in the non-dominant arm. From 7 a.m. to 10 p.m. ambulatory blood pressure was measured at 20 minute intervals and from 10 p.m. to 7 a.m. at 30 minute intervals. To correct for asynchronous day-night patterns between patients, daytime was defined as the period between 9 a.m. and 10 p.m., and nighttime was defined as the period between 1 and 6 a.m. Using these criteria all patients were awake during daytime and asleep during nighttime.

From the hourly averages of ambulatory blood pressure recordings daytime, nighttime and 24-hour averages of systolic, diastolic and mean blood pressure, and heart rate were calculated for each patient.

Statistics

Data are presented as mean ± standard deviation. For comparison of means Student's two-tailed paired t-test was used. A p-value of 0.05 or less was considered to indicate statistical significance.

RESULTS

Clinical characteristics

Twelve male and six female kidney transplant recipients, aged 39 ± 13 yr., were studied. The time after transplantation when the first study was performed ranged from 6 to 89 months (mean 24 months). At the time of the first study the daily CsA dose was 5.4 ± 1.4 mg/kg, resulting in a CsA whole blood trough concentration of 250 ± 66 mg/L. At the time of the second study the mean daily dose of AZA was 1.8 ± 1.4 mg/kg. During both studies each patient used an identical daily dose of prednisone (range 7.5 to 12.5 mg, mean 10.4 mg). Body weight was similar during CsA and AZA. Conversion from CsA to

AZA was not complicated by episodes of graft rejection in the period between both studies.

During CsA all patients were treated with antihypertensives, whereas after conversion to AZA only 4 patients used antihypertensive treatment. The mean number of antihypertensive drugs (calcium channel blockers and/or beta blockers) per patient decreased from 1.3 ± 0.5 before to 0.3 ± 0.6 after conversion ($p<0.05$).

	CsA	AZA	p
Body weight (kg)	76 ± 15	77 ± 15	n.s.
Glomerular filtration rate (ml/min.)	50 ± 14	57 ± 19	p<0.05
Renal blood flow (ml/min)	377 ± 97	431 ± 150	p<0.05
Renal vascular resistance (mmHg/ml/min)	0.346 ± 0.108	0.283 ± 0.116	p<0.05
Renin (μU/ml)	27.7 ± 12.4	34.7 ± 12.2	n.s.
Norepinephrine (nmol/ml)	1.75 ± 0.79	1.81 ± 0.88	n.s.
Atrial natriuretic peptide (pg/ml)	281.5 ± 189.8	210.4 ± 130.9	p<0.05

Table 1. Body weight, renal hemodynamics and hormonal parameters before and after conversion from CsA to AZA.

24-Hour blood pressure recordings

Reliable ambulatory blood pressure recordings were obtained in all patients, allowing to use 92 ± 4% of all readings of the first and 90 ± 8% of the second recordings for analysis. As anticipated, ambulatory blood pressure was higher during CsA than during AZA treatment. The nocturnal decline in blood pressure during CsA, but not during AZA treatment, was almost completely abolished (Fig.1). During CsA only 4 patients had a nocturnal decline of mean blood pressure of more than 10 mmHg, whereas during AZA treatment this number had increased to 9 patients. After conversion, the nocturnal decline in mean blood pressure increased from 3.9 to 9.9 mmHg ($p<0.05$), and the ratio of night-time to day-time mean blood pressure decreased from 0.96 ± 0.08 to 0.91 ± 0.06 ($p<0.01$)(Fig.2). Unlike blood pressure, ambulatory heart rate and its nocturnal decline were similar during both immunosuppressive regimens (Fig.1).

Renal hemodynamics

After conversion RBF and GFR both moderately increased and RVR decreased (Table 1). The nocturnal fall in mean blood pressure or the level of 24-h mean blood pressure were not significantly correlated with neither absolute values of RBF, GFR or RVR nor changes of these parameters after conversion.

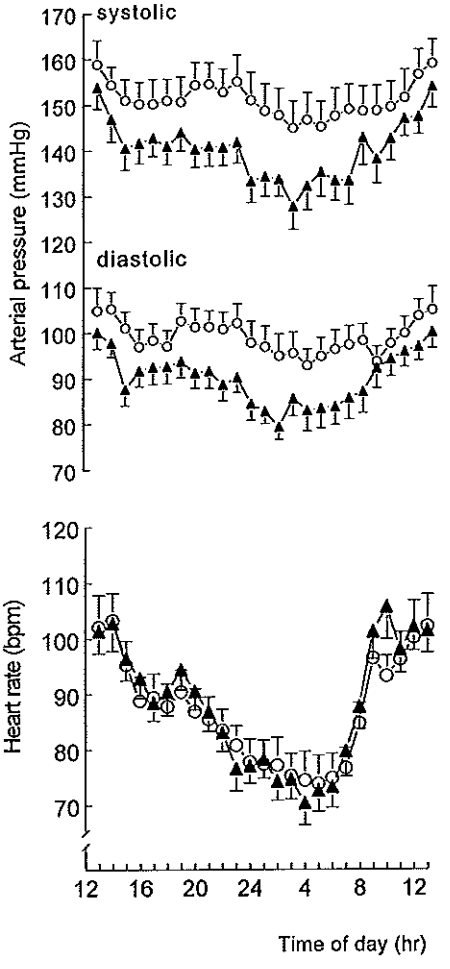

Figure 1. Twenty-four hour systolic and diastolic blood pressure and heart rate during CsA (O) and AZA (Δ). Data are presented as hourly averages ± SEM

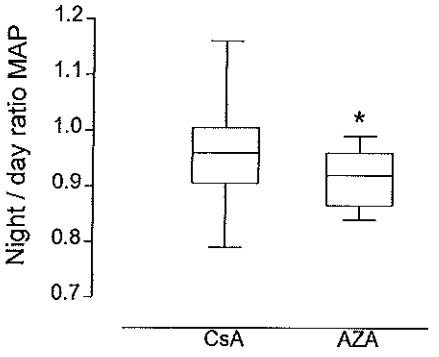

*Figure 2. Box-whisker plots of the ratio between night-time and day-time mean arterial pressure (MAP) of individual patients during CsA and AZA. * indicates p<0.01.*

Vasoactive hormones

Plasma concentrations of renin and norepinephrine were within the normal range of our laboratory. Plasma norepinephrine concentration did not change after conversion, but plasma renin concentration tended to decrease (Table 1). During both immunosuppressive regimens values of plasma ANP concentrations were about twofold increased as compared to the normal reference values of our laboratory. After conversion from CsA to AZA, plasma ANP concentration moderately decreased ($p<0.05$)(Table 1). None of the hormonal parameters was significantly correlated with the decline in nocturnal mean blood pressure or the level of 24-hour MAP.

DISCUSSION

Our study confirms the results of previous studies showing that CsA treatment increases blood pressure in renal transplant recipients (10,12,14). In addition, it shows

that the use of CsA may contribute to the loss of the nocturnal fall in blood pressure in these patients. The ratio between night-time and day-time mean arterial pressure decreased after conversion, indicating that on average a more physiological diurnal blood pressure profile was present during AZA treatment.

The mechanism by which CsA impairs the nocturnal fall in blood pressure is unclear. A decrease in sympathetic tone and increase in vagal tone are considered to be important for the normally occurring nocturnal decline in blood pressure (19). Administration of CsA has been reported to be associated with an increase in muscle sympathetic nerve activity in heart transplant recipients and in patients with myasthenia gravis (10). Therefore, an increase in sympathetic tone might have contributed to the blunted diurnal blood pressure rhythm in our patients as well. However, neither basal plasma norepinephrine concentration, as an approximate index of sympathetic tone, nor the nocturnal decrease in heart rate, as an indirect parameter of sympathetic-vagal balance, were affected by CsA in the present study.

As compared to the AZA based immunosuppressive regimen GFR was moderately reduced and RVR markedly increased during CsA therapy. An attenuation of the nocturnal fall in blood pressure has been observed in patients with advanced renal insufficiency and in patients with end-stage renal disease on hemodialysis (6). This abnormal diurnal blood pressure variation has been linked to the sodium and fluid retention occurring in these conditions. One may wonder whether such a mechanism was also operative in our patients during CsA treatment. Although body weight did not change, the plasma concentration of ANP was higher and the plasma renin concentration tended to be lower during CsA than during AZA treatment, suggesting that some degree of intravascular fluid retention was present during CsA treatment. A significant increase in plasma ANP concentrations in healthy men after a high oral dose of CsA has also been reported by Struthers et al. (14). The elevated plasma ANP concentration may also be related to glucocorticoid-induced sodium retention, that can be hold responsible for the residual non-dipping that was observed in 9 out of 18 patients during AZA. Furthermore, *in vitro* studies have shown that glucocorticoids have the ability to enhance the cardiac release of ANP (20).

A loss of nocturnal fall in blood pressure after organ transplantation has been reported by various groups of investigators (1,2,5,7). It has been suggested that after cardiac transplantation the denervated state of the transplanted heart accounted for this

hemodynamic abnormality, whereas regain of the nocturnal decline in blood pressure was explained by reinnervation (8). Other investigators have reported that the reappearance of the nocturnal decline in blood pressure in heart transplant recipients was correlated to the reduction of the dose of glucocorticoids (5,8). From earlier studies it appears that the nocturnal decline in blood pressure is attenuated or abolished in patients with exogenous or endogenous hypercortisolism (9). Thus, glucocorticoids as well as cardiac denervation seem to be important determinants of the attenuation of the nocturnal decline in blood pressure after heart transplantation. In this study we show that in addition to these factors, the use of CsA is associated with a blunted diurnal blood pressure rhythm. It is still unclear which single factor contributes most to the disturbance of 24-hour blood pressure control in transplant patients.

As a consequence of the treatment protocol CsA therapy was replaced by AZA therapy and not vice versa. Therefore, the patients were always examined in the same order, which might have influenced the outcome of the study. During both recordings 24-hour heart rate profiles were very similar, suggesting that no important differences in sleep quality or night-time physical activity had occurred. Studies on the reproducibility of ambulatory blood pressure recordings have not shown a consistent difference between successive recordings (21-23). If an order effect is present, this usually leads to a slightly higher blood pressure during the initial phase of the first recording. Since in our study the recordings were started at 1 p.m., this effect would have resulted in a larger day-night difference during CsA than during AZA therapy, whereas the opposite was actually found.

In this study the intravascular volume status was assessed by non-invasive measures, which consistently pointed towards volume expansion during CsA. Due to ethical considerations, we were unable to expand our study with more invasive procedures such as cardiac catheterisation or additional radio-isotope studies, which also have particular methodological problems.

In conclusion, we found that in addition to other factors, treatment with CsA per se is associated with a loss of the nocturnal decline in blood pressure in renal transplant recipients. The clinical relevance of our findings remains speculative for the moment. In patients with essential hypertension or secondary forms of hypertension a loss of is diurnal blood pressure variability is associated with an increased incidence of left ventricular hypertrophy and cardiovascular morbidity (24). In renal transplant recipients a close relationship between 24-hour blood pressure and the development of left ventricular

hypertrophy has been found (25). Recently, it has been reported that conversion from CsA to AZA results in improved graft and patient survival and a trend towards a lower cardiovascular mortality (26). Further studies are needed to evaluate the potentially beneficial long-term effects of conversion from CsA to AZA and the relationship between 24-hour blood pressure variation and cardiovascular morbidity and mortality in this patient group.

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Chapter 4

INCREASED LOW DENSITY LIPOPROTEIN OXIDATION IN STABLE KIDNEY TRANSPLANT RECIPIENTS

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SUMMARY

Increased low density lipoprotein oxidation in stable kidney transplant recipients. We aimed to study factors that may add to the high risk of atherosclerosis in kidney transplant recipients. We determined plasma lipoprotein concentrations and parameters of low density lipoprotein (LDL) oxidation in 19 clinically stable kidney recipients and 19 healthy controls. Plasma triglycerides and total cholesterol were increased in the patients. High density lipoprotein-cholesterol (HDL-c) was in the normal range. The mean LDL diameter was smaller in patients than in controls (236 ± 7.1 Å versus 247.8 ± 11.6 Å, $p < 0.002$), which was due to a higher frequency of the LDL subclass pattern B in the patients than in controls (58% versus 28%). The lag time of copper-induced *in vitro* LDL oxidation was shorter in patients than in controls (101 ± 23 min versus 148 ± 81 min, $p = 0.02$). The titre and concentration of autoantibodies against malondialdehyde-modified (MDA-LDL) determined by ELISA were higher in the patients than in the controls. This difference was found in both IgG (titre + 9%, concentration + 75% ($p < 0.05$)) and IgM (titre + 35%, concentration + 102% ($p < 0.001$)).

Based on these results, we propose that there is *in vitro* and *in vivo* evidence of enhanced LDL oxidation in patients post-renal transplantation. This might represent one cause for the clinical finding of advanced atherosclerosis in these patients.

INTRODUCTION

Cardiovascular atherosclerosis is frequently found in patients with chronic renal insufficiency. If atherosclerosis is present before transplantation, it continues to progress after renal transplantation (1). Several factors may contribute to the progression of the atherosclerosis (2). Before transplantation, most patients are suffering from chronic renal failure, which is associated with a high incidence of atherosclerosis (3,4). After transplantation, there is an increased prevalence of the well-established risk factors, hypertension (5,6) and hyperlipidemia (7-9). Besides increased plasma lipoprotein concentrations, alterations in the composition (10) and susceptibility for oxidation (11) of the lipoproteins may also play a role in the atherosclerosis in kidney transplantation patients. Chemical modification of LDL, including oxidation, probably precedes the uptake of LDL by macrophages and the accumulation of cholesterol in the arterial wall (12,13).

The presence of oxidatively modified LDL in atherosclerotic lesions supports this hypothesis (14-16). The susceptibility of LDL for oxidation can be determined *in vitro* (17). As a measure of the susceptibility of LDL for oxidation the time that elapses before lipid peroxidation products become detectable (lag phase), can be taken (17). The susceptibility of LDL for oxidation may be one factor determining LDL oxidation *in vivo*. The lag phase was found to be correlated with the extent of coronary atherosclerosis in humans (18). Human plasma contains autoantibodies against epitopes of oxidized LDL (19,20). The level of these antibodies may also reflect the rate of LDL oxidation (19). Progression of peripheral atherosclerosis correlates with the titre of these antibodies (20).
The lipoproteins in the LDL density range form a heterogeneous population of particles of different sizes (21-24). LDL may be separated by size via gradient gel acrylamide electrophoresis; several LDL subclasses are distinguished in this way (22). If the LDL fraction contains mainly large LDL, this is designated as the LDL subclass pattern A. The presence of mainly small-dense LDL is indicated as the LDL subclass pattern B (25,26). The pattern B is associated with a high plasma triglyceride and a low HDL cholesterol concentration and is, partly, determined by genetic factors (25,26). Subjects with the LDL subclass pattern B have an increased risk of coronary heart disease (25-29). The atherogenicity of small-dense LDL may be due to its association with increased plasma triglycerides and lowered HDL cholesterol. On the other hand, small LDL may play a role in the onset or progression of CHD due to the physical and physiological properties of this LDL. Small LDL has a different carbohydrate composition than large LDL (30). This may lead to an enhanced uptake of small LDL by intima-media (31). In addition, small LDL is more prone to oxidative modification than larger LDL (32,33). Therefore, the LDL subclass pattern, the susceptibility of LDL for oxidation *in vitro* and the level of autoantibodies to epitopes of oxidised LDL seem to be indicators of *in vivo* LDL oxidation. Besides the LDL-cholesterol concentration, these variables may reflect the atherogenicity of the LDL fraction. In this study, we determined these variables in renal transplantation recipients and matched controls.

METHODS

Study population

We studied 19 stable kidney transplant recipients (13 males, 6 females) and 19 controls, matched for sex, age and body mass index. The patients were considered to be

in a stable condition if no rejection episodes or increase in serum creatinine of more than 20 μmol/l had occurred in the 6 months before blood sampling. None of the patients were treated with high doses of corticosteroids during this period. Exclusion criteria were the presence of proteinuria or diabetes. Before transplantation, one patient had been suffering of angina pectoris and one patient of intracerebral haematoma. The other patients had no cardiovascular problems before transplantation. At the time of blood sampling none of the patients showed signs of cardiovascular disease except hypertension. Nine patients were treated with calcium antagonists, nine with labetolol and one with metoprolol. All antihypertensive drugs were discontinued for at least 3 days before blood sampling.
None of the control subjects had overt atherosclerosis or used antihypertensive drugs. Table 1 shows the clinical data of patients and control subjects.

	Patients	Controls
Males (n)	13	13
Females (n)	6	6
Age (y)	42.2 ± 12.3	47.3 ± 9.1
Body mass index (kg/m^2)	24.9 ± 3.5	25.6 ± 3.1
Time after transplantation (m)	24 ± 5	-
Cyclosporin dose (mg/kg/d)	5.5 ± 1.7	-
Prednisone dose (mg/d)	9.6 ± 2.1	
Serum creatinine (μM)	155 ± 44	76 ± 9
Creatinine clearance (ml/min)	59 ± 22	116 ± 29
Systolic blood pressure (mmHg)	149 ± 20	
Diastolic blood pressure (mmHg)	97 ± 14	

Table 1. Characteristics of patients and control subjects.

Blood sampling

Blood samples were drawn into EDTA containing polypropylene tubes (final concentration EDTA, 1 mg/ml) on ice. Plasma was obtained by low speed centrifugation at 4°C. Sucrose was added to the plasma (10 µl sucrose solution per ml plasma) was added to a final concentration of 0.6% to prevent LDL aggregation (34). Plasma was stored at -80°C until use.

Separation of plasma lipoproteins

Low density lipoproteins for oxidation experiments were isolated by density gradient ultracentrifugation as described by Redgrave and coworkers (35). To prevent oxidation of the lipoproteins during ultracentrifugation 0.1 mM EDTA and 0.005% thiomersal were added to the gradient solutions. Before use, the solutions were gassed by nitrogen to remove oxygen. All runs were for 24 h at 15°C and 40.000 rpm in SW 41 TI Beckman rotor using polyallomere tubes. HDL cholesterol was determined after precipitation of apoB containing lipoproteins with heparin/$MnCl_2$ (36). The plasma LDL-cholesterol concentration was calculated using the Friedewald formula (37).

Low density lipoprotein oxidation

Immediately after isolation part (300 µl) of the LDL fraction was placed in a microdialysis apparatus. Six samples were simultaneously dialysed against 120 ml phosphate buffered saline (PBS, 0.15 M NaCl, 0.01 M phosphate, pH 7.4) for 48 h at 4°C in the dark. The dialysis buffer was refreshed after 24 h. The LDL-cholesterol content was measured and the samples were diluted with PBS to a final concentration of 0.25 uM LDL-cholesterol. The LDL oxidation experiments were carried out as described by Esterbauer et al. (17). Six samples of LDL were measured simultaneously in a thermostated Perkin-Elmer Lambda 5 spectrophotometer at 25°C. Oxidation was initiated by the addition of a freshly prepared copper chloride solution (final concentration 1.66 uM). LDL oxidation was followed by monitoring the change in absorbance at 234 nm every two minutes for 16 hr. The lag time was defined as the interval between initiation of the reaction and the intercept of the tangent of the slope of the absorbance curve with the time scale axis expressed in minutes.

LDL size and subclass determination

The LDL subclass patterns were identified by electrophoresis on 2-16% PAGE gels, as described by Austin et al. (26). The gels were prepared with an LKB 11300 Ultrograd gradient mixer (38). In each gel, reference sera with known subclass pattern were applied to lane one and six of a total 12 lanes. The gels were stained with Oil Red O for lipid and the subclass pattern determination. For the determination of the LDL size, a set of standard proteins with known hydrated diameters was run on the same gel as the samples. The standard proteins (HMW electrophoresis calibration kit, Pharmacia, Piscataway, NJ, USA) were thyroglobulin (170 Å), ferritin (122 Å) and catalase (104 Å). The gels were stained with Coomassie Brilliant Blue R 250. The centre of the most prominent LDL band was marked on the gel. The migration distance of the bands from the top of the gel was measured. The average LDL particle diameter was estimated from a quadratic extrapolation of a plot of the logarithm of the diameter of the standards versus the migration distance of the standards (39).

Autoantibodies against malondialdehyde-modified LDL

Malondialdehyde-modified LDL (MDA-LDL) was prepared as described by Palinski et al. (40). Concentrations of autoantibodies against MDA-modified LDL were determined by ELISA (41). The method was modified after the solid-phase radioimmunoassay as described by Salonen et al. (20). Microtiter plates with high binding capacity (Greiner no.655061) were coated with 50 µL MDA-LDL (LDL-cholesterol = 14 - 16 µM) or native LDL at the same concentration in phosphate buffered saline (PBS) (0.15 M NaCl, 0.05 M phosphate, pH 7.4) containing 0.27 mM EDTA and 20 µM butylated hydroxytoluene (BHT) for 2 hr at 37°C. After incubation, each plate was washed with PBS containing 0.05% Tween-20 and 0.001 % aprotinin using a microplate washer (Biorad model 1550). The remaining binding sites were blocked with 150 µl 2% bovine serum albumin (BSA) in PBS for 2 hr at room temperature. The BSA-solution was heated at 56 °C for 30 min, filtered over a paper filter and cooled to room temperature before use. Each plate was washed as described above. Duplicate plasma samples in dilution of 1/1667 for IgM class and 1/833.3 for IgG class, in a total volume of 50 µl, were added to the wells and incubated overnight at 4 °C. Wells not incubated with MDA-LDL were used as a blank. The next day the wells were aspirated and washed again as described above. Fifty µl of a thousand-fold

diluted monoclonal mouse antibody against human IgM or IgG (Sigma Immunochemicals, St. Louis, USA) in PBS was added and the plates were incubated for 4 hr at 4 °C and subsequently washed as described above. A peroxidase conjugated goat anti-mouse antibody (Tago Inc., Burlingname, California, USA) 50 µl was added to the wells in a thousand-fold dilution in PBS and incubated for one hr at 37 °C, followed by the washing procedure. For the substrate reaction, an orthophenylenediamine dihydrochloride solution (2 mg/ml) (Sigma Chemical Co., St. Louis, USA) in citric acid (0.1 M) phosphate (0.2 M) buffer (pH 5.5), containing 0.015% hydrogenperoxide was prepared just before use. Fifty µl of the substrate solution was added to each well and incubated in the dark during exactly 20 minutes. The reaction was stopped with 50 µl 2.5 M H_2SO_4 per well.

Absorbance was measured using a microplate reader (Biorad model 450) at 490 nm. The absorbance was linear with the amount of diluted plasma added up to 100 µl. The antibody titre was defined as the absorbance of the wells coated with MDA-LDL divided by the absorbance of the wells coated with native LDL for each plasma sample (20). The concentration of MDA specific autoantibodies was calculated from the difference in absorbance between the MDA-LDL-coated wells and native-LDL-coated wells for each sample. The absorbances were converted in µg antibody/ml plasma by comparison with a standard curve of a pool plasma with known anti-MDA-LDL antibody concentrations (IgM 27.0 ± 2.0 µg/ml, IgG 5.8 ± 0.1 µg/ml)(41).

Other analytical methods

Plasma cholesterol and triglycerides (Boehringer Mannheim, Mannheim, Germany) and creatinine (Sigma Diagnostics, St. Louis, USA) were determined using commercially available test kits. An estimate of the creatinine clearance rate was calculated from the plasma creatinine concentration as described by Cockcroft and Gault (42).

Statistical analysis

Data are presented as means ± standard deviation. Differences between groups were evaluated for significance using the Student-t-test or by ANOVA followed by Bonferoni for comparison of groups. Simple correlations between variables were calculated using the Pearson correlation test. The level of significance was set at $p < 0.05$.

RESULTS

Lipids and lipoproteins in renal transplant recipients and controls

Plasma triglyceride and cholesterol levels were significantly higher in renal transplant recipients than in controls (68% and 12%, respectively, Table 2). In 47% (9/19) of the patients, cholesterol was > 6.2 mM (240 mg/dl), while in the control group only one subject had a cholesterol of 6.2 mM. LDL-cholesterol tended to be higher in the patients than in the controls (+ 8%, p = 0.13). In 37% (7/19) of the patients and in one control LDL-cholesterol was > 4.0 mM (155 mg/dl). The mean HDL cholesterol in the patients was not different from controls.

	Patients	Controls	p
Total cholesterol	5.91 ± 0.95	5.33 ± 0.50	0.017
Total triglyceride	2.40 ± 0.99	1.43 ± 0.65	0.001
HDL-cholesterol	1.09 ± 0.39	1.21 ± 0.23	0.28
LDL-cholesterol	3.73 ± 0.70	3.44 ± 0.44	0.13

Table 2. Lipids and lipoproteins in renal transplant recipients and controls. All variables are in mM ± standard deviation.

LDL size and subclass pattern

The mean size of the most prominent LDL fraction was significantly less in the patients than in controls (Table 3). The size of LDL was inversely correlated with the plasma triglyceride (r = -0.66, p < 0.001) and weakly positively with HDL cholesterol (r = 0.34, p < 0.05). The smaller size of the LDL particles was reflected in the LDL subclass pattern. The LDL subclass pattern B, with a mean diameter of the major LDL subfraction of 233.3 ± 3.1 Å, was more frequently found in the patients (58%) than in the controls (29%)(Pearsons chi-square test, p = 0.028, Table 3). The LDL subclass pattern A (mean particle diameter 250.9 ± 9.2 Å) was present in 26% of the patients and in 68% of the controls. The mean size of the major LDL fraction in the LDL subclass pattern A and B in controls and patients were not significantly different (controls versus patients, pattern A

253.4 ± 9.2 Å versus 245.6 ± 6.1 Å, pattern B 235.9 ± 3.6 Å versus 232.1 ± 2.2 Å). An intermediate LDL subclass pattern was found in two patients and in one control.

The LDL subclass pattern B was associated with a higher plasma triglyceride and a lower HDL cholesterol (Table 4). Considering a plasma triglyceride above 2.3 mM and HDL-cholesterol beneath 0.9 mM as abnormal, in 7 patients small-dense LDL together with

	Patients	Controls
Number	19	19
LDL size (Å)	236.5 ± 7.3*	247.8 ± 11.6
LDL subclass		
Pattern A	6 **	13
Pattern A/B	2	1
Pattern B	11 **	5

*Table 3. Low density lipoprotein size and subclass patterns in kidney transplant patients and controls. *,** Significantly different from controls (* student-t-test, p < 0.002, ** Chi-square test, p = 0.028).*

decreased HDL cholesterol and increased plasma triglyceride was found, in contrast to the control group in which only one subject met these criteria. LDL-cholesterol was similar among subjects with different LDL subclasses. There was no relation between LDL size and LDL-cholesterol or total plasma cholesterol (not shown).

	Pattern A	Pattern A/B	Pattern B
Total triglyceride	1.39 ± 0.58	1.75 ± 0.21	2.60 ± 1.00*
Total cholesterol	5.40 ± 0.74	5.40 ± 0.40	5.91 ± 0.87
LDL-cholesterol	3.52 ± 0.53	3.51 ± 0.61	3.68 ± 0.69
HDL-cholesterol	1.24 ± 0.26	1.07 ± 0.08	1.06 ± 0.39

*Table 4. Lipids and lipoproteins in subjects with different LDL subclass patterns. All variables are in mM. * denotes a statistically significant difference between LDL subclass pattern A and B (* p < 0.001).*

Low density lipoprotein oxidation

The susceptibility of LDL to oxidation was determined by following *in vitro* Cu^{2+}-induced LDL oxidation. In the kidney recipients the lag time was 32% shorter than in controls (Table 5). In subjects with an LDL-subclass pattern B the lag phase was significantly shorter than in subjects with an LDL-subclass pattern A (- 38%)(Table 5). In patients and controls with the same subclass pattern, the lag time tended to be shorter in the patients but the differences were not statistically significant (Table 5). There was no correlation between plasma LDL-cholesterol and lag time in neither controls nor patients (not shown).

LDL-subclass	All	Patients	Controls
All	124 ± 63 (38)	101 ± 23 (19)	148 ± 81 (19)*
Pattern A	154 ± 78 (19)[a]	122 ± 30 (6)	169 ± 80 (13)
Pattern A/B	94 ± 10 (3)	94 ± 15 (2)	97 (1)
Pattern B	95 ± 15 (16)[b]	92 ± 11 (11)	102 ± 21 (5)

*Table 5. Lag time of low density lipoprotein oxidation in vitro. Given are the lag times in min. Between brackets the number of samples is shown. [a] is significantly different from [b] (ANOVA followed by Bonferoni, $p < 0.02$). * denotes a statistically significant difference between patients and controls ($p = 0.02$)*

Autoantibodies against MDA-modified LDL

Concentrations and titres of autoantibodies against MDA-LDL were significantly higher in renal transplant patients than in controls (Table 6). IgM autoantibody concentrations in the patients were on the average about 2-fold higher than in the controls. IgG autoantibodies were 75% higher in the renal transplant group. The titre of the IgM autoantibodies against MDA-LDL was 35% and of the IgG autoantibodies 9% higher in the patients than in the controls, both being statistically significantly different (Table 6). In patients with an LDL-subclass pattern B IgM antibody values were higher than in subjects with the pattern A, IgM antibodies pattern B versus pattern A 48.3 ± 17.6 μg/ml versus 26.2 ± 10.0 μg/ml ($p < 0.001$), IgM antibody titres 2.11 ± 0.35 versus 1.58 ± 0.21 ($p < 0.001$). Pattern A and pattern B subjects did not differ in IgG autoantibodies. There was no correlation between LDL-cholesterol and any of the antibody parameters nor between the plasma CsA content and any of the determined variables (not shown).

DISCUSSION

In kidney transplant recipients varying degrees of hyperlipidemia have been reported (2,43-48), HDL-cholesterol being lowered, enhanced or unaffected (10,47,48). Our study group, which consisted of patients in a stable condition, resembled the average lipid profile in kidney transplant patients with an increased plasma cholesterol (> 6.2 mM (240 mg/dl)) in 47% of the patients. Since plasma triglyceride was elevated part of the hypercholesterolemia was attributable to an increase in VLDL- cholesterol. Still 37% of the patients had an LDL-cholesterol above 4 mM (155 mg/dl). Kidney transplantation is associated with an increased occurrence of atherosclerosis. Several factors are associated with the atherosclerosis, hypercholesterolemia being one of them (2). During the past years it has become clear that besides an enhanced LDL cholesterol other lipoprotein-associated factors are related with atherosclerotic risk. Oxidation of LDL is considered as a major event in the development of atherosclerosis. We found that parameters of *in vitro* (lag phase of LDL oxidation) and *in vivo* (autoantibodies against

	Patients	Controls	p
Antibody concentration			
IgM (μg/ml)	49.4 ± 15.9	24.4 ± 8.7	< 0.0001
IgG (μg/ml)	5.6 ± 3.8	3.2 ± 1.9	< 0.02
Antibody titre			
IgM	2.10 ± 0.32	1.56 ± 0.22	< 0.0001
IgG	1.25 ± 0.19	1.15 ± 0.10	< 0.05

Table 6. Autoantibodies against MDA-LDL in renal transplant patients and controls.

MDA-LDL) LDL oxidation are greatly affected in renal transplant patients, indicating that LDL oxidation may be increased in these patients. We, therefore, think that one cause of the aggravated atherosclerosis in renal transplant patients may be an increased LDL oxidation. Recently, Maggi and coworkers reported enhanced levels of autoantibodies

against MDA-LDL in patients during chronic haemodialysis (49). During this treatment, which often precedes transplantation, atherosclerosis is probably initiated. After transplantation, the atherosclerosis may progress even more. The rate of *in vivo* LDL oxidation is presumably dependent on several factors. Renal insufficiency seems to be not a major determinant of the LDL oxidation as none of the oxidation parameters were correlated with creatinine clearance in patients or controls. The susceptibility of LDL for oxidation, which is reflected in the lag phase of copper-induced *in vitro* LDL oxidation, is probably an important factor determining the *in vivo* LDL oxidation rate. The LDL size is one determinant of its susceptibility for *in vitro* oxidation, small-dense LDL being more susceptible than larger LDL (32,33). This was also found in the patients and controls in the present study. Another factor influencing the LDL oxidation in transplant patients may be the use of cyclosporin A. Apanay and coworkers (11) found in renal transplant patients with a high plasma cyclosporin level shorter lag phases than in controls and in patients with lower cyclosporin levels. Moreover, an inverse correlation between the amount of LDL-associated cyclosporin and the lag phase was found to exist. We found no correlation between the lag phase and the plasma cyclosporin level although the lag phases of the renal transplant recipients tended to be shorter if compared to controls with the same LDL subclass. Therefore, cyclosporin may have a small effect on the lag phase, but it seems that the difference in the mean LDL oxidation lag time between control and patient groups is mainly due to the high number of subjects with small LDL in the renal transplant group. Recently, we demonstrated that, in subjects with coronary artery disease, autoantibodies against MDA-LDL were higher in patients with the LDL subclass pattern B than with the LDL subclass pattern A (41). In the renal transplant patients this was also the case. This suggests that not only *in vitro*, but also *in vivo*, LDL of patients with the LDL subclass pattern B is more readily oxidised than larger LDL. In this respect it is of interest that Taylor and coworkers (50) showed that the oxidation potential is higher in renal transplant recipients than in controls as evidenced by higher plasma malondialdehyde levels, lower plasma thiols and increased red cell superoxide dismutase. Thus several factors may add to an enhanced LDL oxidation in the patients, a larger number of patients with small-dense LDL, increased susceptibility for oxidation by cyclosporin and a higher overall oxidation potential. The presence of small-dense LDL may be, at least, partly metabolically explained. The LDL subclass pattern B is associated with an increased (VLDL)triglyceride and low HDL-C (38,51). In our study also a lower LDL size was

associated with higher plasma triglyceride and lower HDL cholesterol levels. If the lowering in LDL size is due to the increase in triglycerides, the increased plasma triglyceride level may be one cause of enhanced LDL oxidation. Supposing that increased LDL oxidation contributes to the high atherosclerotic disease in the transplant patients, a lowering of plasma triglycerides (if it leads to a shift of LDL subclass pattern and subsequently less LDL oxidation) may help to diminish the progression of atherosclerosis in renal transplant patients.

In conclusion, our results show that in renal transplant recipients, there is increased incidence of the LDL subclass pattern B with an increased susceptibility to oxidative modification. Probably, LDL oxidation *in vivo* is also increased in the transplant patients as indicated by increased values of autoantibodies against MDA-LDL. These factors may play a role in the accelerated atherogenesis occurring after renal transplantation.

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Chapter 5

CONVERSION FROM CYCLOSPORIN A TO AZATHIOPRINE TREATMENT IMPROVES LDL OXIDATION IN KIDNEY TRANSPLANT RECIPIENTS

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ABSTRACT

Oxidative modification of LDL plays an important role in the initiation and progression of atherosclerosis. There is evidence that CsA may facilitate lipid peroxidation *in vitro* and *in vivo,* and thereby contribute to the high incidence of cardiovascular disease after transplantation.

We determined several parameters of LDL oxidizability in 19 renal transplant recipients both before and after conversion from CsA to azathioprine. The susceptibility of LDL to *in vitro* oxidation, LDL particle size, plasma titres of IgG and IgM antibodies against oxidised LDL and plasma LDL subclass patterns were measured. In addition, arterial pressure was recorded, and renal hemodynamcis were estimated from the clearance of radiolabeled thalamate and hippurate.

After conversion, the plasma concentrations of total cholesterol, LDL-cholesterol and triglyceride decreased, while plasma HDL-cholesterol did not change.
During CsA therapy the oxidizability of plasma LDL was increased, as reflected by a longer lag phase during *in vitro* oxidation. In addition, LDL size increased and the titres of IgM- and IgG-autoantibodies against oxidised LDL decreased significantly upon conversion. The more atherogenic LDL subclass pattern B was present in 13 out of 19 patients during CsA. In 5 patients, pattern B changed into pattern A after conversion. The subclass B pattern was maintained in 8 patients and subclass A pattern in 6 patients.

Our study demonstrates that treatment with CsA increases the susceptibility of LDL to *in vitro* oxidation and also enhances the oxidation of LDL *in vivo.* In combination with lower arterial pressure, better renal function and a more favourable lipid profile, the changes in lipid peroxidation may contribute to the cardiovascular complications during CsA treatment.

INTRODUCTION

The use of cyclosporine A (CsA) has significantly improved graft survival after organ transplantation (1). Unfortunately, patients treated with CsA often develop hypertension and hyperlipidemia (2-6). These factors may contribute to the development of cardiovascular disease, which is the most important cause of death after renal transplantation (7). It has been found that the use of CsA is associated with a more atherogenic plasma lipid profile (4, 8, 9). CsA raises plasma cholesterol concentration, mainly by increasing the plasma LDL-cholesterol fraction, without affecting plasma

HDL-cholesterol (3, 8). CsA also influences lipoprotein properties and appears to increase lipid peroxidation (10). The susceptibility of low density lipoproteins to oxidation is also increased by CsA, the lag phase of *in vitro* LDL oxidation being inversely correlated with the amount of CsA in LDL (11). Moreover, in kidney transplant recipients on CsA the prevalence of small-dense LDL (corresponding with LDL subclass pattern B), which is more susceptible for oxidation than large LDL (corresponding with LDL subclass pattern A), is increased (12). Since Taylor and coworkers demonstrated that overall lipid peroxidation is increased in kidney transplant recipients on CsA (13), these factors together may promote LDL oxidation *in vivo*. This is also suggested by the presence of increased autoantibodies against malondialdehyde-LDL in kidney transplant recipients on CsA (12). Oxidative modification of LDL is thought to play a crucial role in the onset and progression of atherosclerotic disease (14). Oxidized LDL contains novel epitopes, which act as chemoattractants for monocyte and macrophage migration, thus stimulating the formation of foam cells (15). Oxidized LDL appears to be toxic for endothelial cells. Galle and coworkers found that after acute dosing of CsA to rats, the development of CsA-mediated renal dysfunction was associated with lipid peroxidation (16). Furthermore, oxidized low density lipoproteins inhibit the activity of inducible NO-synthase inactivated macrophages *in vitro* (17,18). This reduction in NO production may influence cell-to-cell interactions and vasomotor tone. In previous studies, conversion from CsA to azathioprine (AZA)-based immunosuppression led to reduction of plasma triglyceride and cholesterol levels (19). As plasma triglycerides are strongly related to LDL size and LDL size is related to the susceptibility of LDL to oxidation, LDL oxidation may also be influenced during conversion from CsA to AZA.

To investigate the effects of conversion of CsA to AZA on lipid peroxidation, we determined plasma lipid profiles and parameters of *in vitro* and *in vivo* LDL oxidation in renal transplant recipients before and 16 weeks after discontinuation of CsA and replacement by AZA. In addition, renal hemodynamic parameters and arterial pressure were measured on both occasions.

METHODS

Patients

Patients were recruited among renal transplant recipients who were enrolled in a prospective randomized clinical trial, which was designated to evaluate the effects of two

different immunosuppressive regimens, i.e. prednisone combined with CsA or with AZA, on long-term graft function and incidence of rejection episodes. For this trial all CsA-treated renal transplant recipients, between 18 and 65 years of age, who were 6 months or longer after transplantation, were randomly allocated to either continuation of CsA treatment, or conversion from CsA- to AZA-based immunosuppression. Patients with diabetes mellitus, proteinuria over 3 grams per day, previous acute graft rejection, or histological evidence of chronic graft rejection were excluded. The first 19 consecutive patients fulfilling these criteria and willing to give written informed consent were studied.

Study design

The first study was performed while patients were on CsA and the second study 16 weeks later, when patients were on AZA therapy. During both studies the patients used the same dose of prednisone. All antihypertensive medication (betablockers in 13 patients and calciumchannel blockers in 8 patients) was discontinued 3 days prior to both study sessions. After the first study AZA was started at a dose of 2 mg/kg daily. Two weeks later the CsA dose was reduced to 50% and the prednisone dose was increased to 30 mg daily. After another two weeks CsA was withdrawn and the prednisone was tapered to the baseline dose within 3 weeks. If necessary, AZA dose adjustments were made according to the white blood cell count and hematocrit.

On both study days patients arrived in the cardiovascular research laboratory after an overnight fast. At arrival they were weighted and subsequently a small catheter (Venflon, Viggo Spectramed, Helsingborg, Sweden) was inserted in a forearm vein of each arm. One catheter was used for the infusion of radio-labeled thalamate and hippuran for renal function studies and the other catheter was used for blood sampling. The study protocol was approved by the local Medical Ethics Committee.

Measurements

Renal hemodynamics

Effective renal plasma flow (ERPF) and glomerular filtration rate (GFR) were estimated from the clearance of ^{131}I-hippuran and ^{125}I-thalamate as desribed earlier (20). During the renal function studies arterial pressure was measured by an automated oscillometric device (AccuTorr2, Datascope Corp., Montvale, NJ, USA). Renal blood flow (RBF) was calculated by dividing effective renal plasma flow by (1- hematocrit). Before the

start of the renal function studies a blood sample was taken for the determination of CsA 12-h trough blood levels (CycloTrac SP, IncStar Corp., Stillwater, MN, USA).

Blood sampling

Blood samples were drawn into EDTA-containing polypropylene tubes (final concentration EDTA, 1 mg/mL) on ice. Plasma was obtained by low speed centrifugation at 4^0C. To the plasma, sucrose (10 μL sucrose solution per mL plasma) was added to a final concentration of 0.6% to prevent LDL aggregation (21). Plasma was stored at - 80°C until use.

Separation of plasma lipoproteins

Low density lipoproteins were isolated by density gradient ultracentrifugation as described by Redgrave et al. (22). To prevent oxidation of the lipoproteins during ultracentrifugation 0.1 mM EDTA and 0.005% thiomersal were added to the gradient solutions. Before use, the solutions were gassed by nitrogen to remove the oxygen. All runs were for 24 hours at 15°C and 40.000 rpm in an SW 41 TI Beckman rotor using polyallomere tubes. HDL cholesterol was determined after precipitation of apoB containing lipoproteins with heparin/$MnCl_2$ (23). The plasma LDL cholesterol concentration was calculated using the Friedewald formula (24).

Low density lipoprotein oxidation

LDL oxidation experiments were carried out using the procedure of Esterbauer (25) monitoring the generation of lipid peroxidation products in the presence of 1.66 mM $CuCl_2$ at 254 nm, as described before (12). The lag time was defined as the interval between initiation of the reaction and the intercept of the tangent of the slope of the absorbance curve with the time scale axis expressed in minutes.

LDL size and subclass determination

The LDL subclass patterns were identified by electrophoresis on 2-16% PAGE gels, as described by Austin et al (26). The gels were prepared with an LKB 11300 Ultrograd gradient mixer (27). In each gel, reference sera with known a subclass were applied to lane one and six of a total 12 lanes. The gels were stained with Oil Red O for lipid and the subclass pattern determination. For the determination of the LDL size, a set of standard

proteins with known hydrated diameters was run on the same gel as the samples. The standard proteins (HMW electrophoresis calibration kit; Pharmacia, Piscataway, NJ, USA) were thyroglobulin (170 Å), ferritin (122 Å) and catalase (104 Å). The gels were stained with Coomassie Brilliant Blue R 250. The centre of the most prominent LDL band was marked on the gel. The migration distance of the bands from the top of the gel was measured. The average LDL particle diameter was estimated from a quadratic extrapolation of a plot of the logarithm of the diameter of the standards versus the migration distance of the standards (28). The subclass pattern A was assigned to LDL particles with a diameter > 238 nm and the subclass pattern B to LDL particles with a diameter < 238 nm. The cutoff level was set at 238 nm, because this produced the best separation of the two subclasses on the gels after electrophoresis (29).

Autoantibodies against malondialdehyde-modified LDL

Autoantibodies against MDA-modified LDL were determined by ELISA as described elsewhere (29). The antibody titer was defined as the absorbance of the wells coated with MDA-LDL divided by the absorbance of the wells coated with native LDL for each plasma sample (30).

Other analytical methods

Plasma cholesterol and triglycerides (Boehringer Mannheim, Mannheim, Germany) and creatinine (Sigma Diagnostics, St. Louis, MO, USA) were determined using commercially available test kits.

Statistical analysis

All data are presented as means ± SD. Data before and after conversion were compared by a paired Student-t-test. Correlations between variables were calculated using the Pearson correlation test. The level of significance was set at $P < 0.05$.

RESULTS

Patient characteristics

The clinical characteristics of the patients during both treatment periods are shown in Table 1.

	CsA	AZA	p
Male / female	13 / 6		
MAP[a] (mmHg)	116 ± 14	109 ± 12	<0.01
Post-Tx time (months)	24 ± 5	27 ± 5	
Body weight (kg)	75.7 ± 15.4	76.9 ± 15.2	n.s.
Body mass index (kg/m^2)	24.9 ± 3.5	24.9 ± 3.3	n.s.
Prednisone dose (mg/day)	9.6 ± 2.1	9.6 ± 2.1	n.s.
CsA dose (mg/kg/day)	5.5 ± 1.7	-	
CsA 12-h trough level (ng/ml)	250 ± 66	-	
AZA dose(mg/kg/day)	-	1.8 ± 0.4	
GFR[b] (ml/min)	50 ± 14	57 ± 19	<0.05
RBF[c] (ml/min)	377 ± 97	431 ± 150	<0.05
RVR[d] (mmHg/ml/min)	0.346 ± 0.108	0.283 ± 0.116	<0.05
Antihypertensives per patient	1.3 ± 0.7	0.3 ± 0.6	<0.05

Table 1. Clinical characteristics of 18 renal transplant recipients before and after conversion from CsA to AZA;
[a]mean arterial pressure; [b] glomerular filtration rate/m^2 body surface area; [c] renal blood flow; [d] renal,vascular resistance.

Effect of conversion on plasma lipids and lipoproteins.

After conversion total cholesterol, LDL-cholesterol and plasma triglycerides decreased significantly. Plasma HDL-cholesterol concentration was stable (Table 2).

	CsA	AZA	p
Total cholesterol (mmol/l)	5.9 ± 0.9	5.0 ± 0.7	<0.005
LDL-cholesterol (mmol/l)	3.7 ± 0.7	3.0 ± 0.6	<0.05
HDL-cholesterol (mmol/l)	1.1 ± 0.4	1.2 ± 0.4	n.s.
Total triglyceride (mmol/l)	2.4 ± 1.0	1.7 ± 0.6	<0.001

Table 2. Plasma lipid concentrations before and after conversion from CsA to AZA.

	CsA	AZA	p
LDL particle size (nm)	236.5 ± 7.3	240.7 ± 6.8	<0.0001
LDL subclass pattern A/B	6 / 13	11 / 8	n.s.
Lag time (min)	98.9 ± 24.3	114.7 ± 17.3	<0.05
IgG anti-MDA-LDL titre	1.24 ± 0.15	1.19 ± 0.14	<0.05
IgM anti-MDA-LDL titre	2.02 ± 0.29	1.78 ± 0.19	<0.0001

Table 3. Parameters of in vitro and in vivo lipid peroxidation before and after conversion from CsA to AZA.

Effect of conversion on LDL-particle size and subclass distribution

The mean diameter of LDL particles increased significantly (236.5 ± 7.3 nm versus 240.7 ± 6.8 nm, p<0.0001). The number of patients which had LDL subclass pattern A, representing the less oxidisable large-size LDL, increased from 6 to 11 out of 19 patients. None of the patients changed from subclass A to subclass B, or had a smaller LDL-size after conversion. The changes of plasma lipid concentrations were closely related to the LDL subclass pattern before conversion. In patients whose subclass pattern changed from B to A, the LDL-cholesterol, total cholesterol and triglycerides fell much more than in patients who had pattern A or B both before and after conversion.

Low density lipoprotein oxidation.

The mean lag time to copper-induced oxidation increased from 99±24 min during CsA, to 115±17 min during AZA, indicating that during AZA, LDL was significantly less susceptible to *in vitro* oxidation. The largest increase in lag time upon conversion occurred in patiemts whose subclass pattern changed from B to A. When the LDL subclass pattern did not change, the lagtime remained stable (pattern A to A), or increased slightly (pattern B to B)(Table 5).

Autoantibodies against MDA-modified LDL

The concentration and titer of IgM autoantibodies against MDA-LDL fell significantly after conversion (-27% and -16% respectively). Both before and after conversion patients with subclass A had a lower IgM antibody concentration than patients with subclass B. The largest decrease in antibody concentration occurred in patients in

	LDL subclass change upon conversion		
	A to A	B to A	B to B
Number of patients	6	5	8
Triglyceride (mM)			
CsA	1.96 ± 0.44	1.94 ± 0.55	2.96 ± 1.22
AZA	1.74 ± 0.52	1.07 ± 0.13	2.17 ± 0.59
p value	0.14	0.02	0.05
Total cholesterol (mM)			
CsA	5.72 ± 1.02	6.28 ± 0.69	6.00 ± 0.94
AZA	4.93 ± 0.89	4.44 ± 0.67	5.14 ± 0.61
p value	0.21	0.003	0.09
LDL cholesterol (mM)			
CsA	3.60 ± 0.70	4.23 ± 0.51	3.50 ± 0.69
AZA	3.15 ± 0.62	2.66 ± 0.64	3.08 ± 0.56
p value	0.27	0.003	0.20
HDL cholesterol (mM)			
CsA	1.23 ± 0.33	1.16 ± 0.51	0.98 ± 0.38
AZA	1.38 ± 0.51	1.30 ± 0.53	1.08 ± 0.37
p value	0.53	0.66	0.58

Table 4. Relationship between the plasma concentration of total cholesterol, LDL- and HDL-cholesterol and triglycerides and the change in LDL subclass pattern following conversion from CsA to AZA.

which the LDL subclass pattern changed from B to A (54.9±18.5 vs. 32.1±8.4 μg/ml, $p<0.01$). The antibody concentration dropped also in the patients in which the LDL subclass pattern A did not change (34.7 ± 8.5 vs. 23.4 ± 4.5 μg/ml, $p<0.05$). In patients with a subclass B pattern before and after conversion, IgM antibody concentration was elevated and remained unchanged (48.7 ± 9.1 vs. 41.8 ± 11.6 μg/ml)(Table 6).

LDL subclass change	Number of patients	Lagtime CsA	Lagtime AZA	p
A to A	6	122 ± 30	131 ± 19	0.51
B to A	5	84 ± 18	115 ± 10	0.01
B to B	8	90 ± 11	102 ± 10	0.04

Table 5 Relationship between the lag time of copperchloride-induced in vitro LDL oxidation and the change in LDL subclass pattern following conversion from CsA to AZA.

DISCUSSION

Cardiovascular disease is the most important cause of death in renal transplant recipients (7). This may be related to pretransplant renal replacement therapy, the underlying renal disease which may be accompanied by elevated blood pressure, or to other factors. Elevated serum cholesterol and hypertension are well-known side-effects of CsA, which occur in a significant proportion of patients. Therefore, the use of CsA may worsen the cardiovascular risk profile of transplant patients.

Recently, it has become clear that the atherogenicity of LDL is, apart from its plasma concentration, dependent on chemical modifications such as oxidation (14,15). Oxidized LDL particles are avidly taken up by scavenger receptors which are present on endothelial cells and macrophages and may induce foam cell formation *in vivo* (32). Oxidized LDL may trigger immune reactions in the vessel wall, promote gene expression of cell adhesion molecules in arterial cells and impair endothelial vasodilator function (32-34).

Our study demonstrates that conversion from CsA to AZA leads to a longer lag time of *in vitro* LDL oxidation, indicating that LDL particles are less susceptible to oxidative modification during AZA. *In vitro* studies have shown that CsA induces lipid peroxidation

both in liver and renal microsomes (10). Recently it has been demonstrated that the oxidizability of LDL is strongly correlated to CsA blood concentrations, suggesting that CsA may facilitate LDL oxidation (11). Increased lipid peroxidation was also found in CsA-treated heart transplant recipients (37). In our study there was no significant correlation between CsA 12 - h trough blood concentrations and any of the lipid peroxidation parameters. The observed effects on LDL oxidation may, apart from a direct influence of CsA, be caused by the increase in LDL size following conversion. It has been shown that small LDL particles, corresponding with pattern B, are more susceptible to oxidation than large LDL particles, which correspond with pattern A (36). Part of our patients shifted from pattern B to A, while none of them shifted from pattern A to B. Therefore, besides the loss of the pro-oxidant effect of CsA, the significant increase in LDL size may have contributed to the decrease in LDL oxidizability *in vitro*.

It is well known that patients with an elevated plasma triglyceride are much more likely to express LDL subclass pattern B and have small-dense LDL particles (36,39). We also found that patients with a subclass B pattern had a higher plasma triglyceride level and had the largest decrease after conversion. The increased plasma triglyceride concentrations associated with the use of CsA may therefore be an alternative explanation for the changes in lipid peroxidation we have found.

The oxidation of LDL *in vivo* is reflected by the presence of autoantibodies against oxidized LDL (38). We found a significant decrease in plasma concentration of IgM antibodies against oxidatively modified LDL following conversion, especially in patients who had an increase of the lag time to *in vitro* LDL oxidation. This suggests that during CsA LDL is more susceptible to oxidation, which may lead to increased LDL oxidation *in vivo*. One may argue that AZA may directly impair antibody production. However, in a previous study we have found that during CsA the production of antibodies against influenza vaccination was inhibited, rather than during AZA (40).

The increased susceptibility to oxidation during CsA was accompanied by a reduction of renal blood flow and glomerular filtration rate. Recently it has been reported that in uninephrectomized rats treated with different doses of CsA, lipid peroxidation products in renal tissue homogenates and renal vascular resistance were dose-dependently increased. Both could be prevented by the coadministration of the antioxidant vitamin E, suggesting that oxidative processes play a causative role in the pathogenesis of CsA-induced renal injury (41). However, other investigators were unable to confirm these

findings in humans (42). In a cross-sectional study Maggi et al. showed that in hemodialysis patients the titre of autoantibodies directed against oxidatively-modified LDL is increased when compared to healthy controls, suggesting that enhanced LDL oxidation occurs *in vivo* (43). In our study no relationship between absolute values or changes in renal function, in blood pressure or duration of pretransplant renal replacement therapy and any of the lipid peroxidation parameters was present.

Only a few of our study patients developed symptomatic cardiovascular disease during the posttransplant period. Obviously, our study population was too small to allow definitive conclusions about the association between the prevalence of cardiovascular events and lipid peroxidation *in vivo.*

In conclusion, this study shows that during CsA when compared to AZA the susceptibility of LDL to *in vivo* and *in vitro* oxidation appears to be increased. In view of recent reports of the importance of oxidatively modified LDL in the pathogenesis of atherosclerosis, our findings offer an additional explanation for the high cardiovascular morbidity and mortality after renal transplantation. The molecular mechanism of our observations is still unresolved. Further studies of the effects of CsA on lipid peroxidation and its implications for atherogenesis are needed.

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Chapter 6

BENEFICIAL EFFECTS OF CONVERSION FROM CYCLOSPORINE TO AZATHIOPRINE ON FIBRINOLYSIS IN RENAL TRANSPLANT RECIPIENTS.

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ABSTRACT

Cyclosporine A (CsA) has been implicated as one of the factors contributing to the high cardiovascular morbidity and mortality following renal transplantation. This may be mediated by either a high prevalence of conventional risk factors for atherosclerosis, such as hypertension, hypercholesterolemia and diabetes mellitus, or by impairment of the fibrinolytic activity evoked by CsA, possibly through interference with prostanoid metabolism.

We therefore assessed the impact of conversion of cyclosporine A to azathioprine immunosuppressive treatment on parameters of fibrinolytic activity and plasma concentration of the prostanoids prostaglandin E_2 (PGE_2) and thromboxane B_2, (TxB_2) in 18 stable renal transplant recipients.

During CsA, mean arterial pressure and serum creatinine were significantly higher than during azathioprine (AZA)(116 ± 15 mmHg vs. 106 ± 13 mmHg, p=0.0003, and 147 ± 34 μmol/l vs. 127 ± 35 μmol/l, p=0.002, mean ± SD). Upon conversion, the plasma tissue plasminogen activator activity increased from 1.22 (0.84-1.95, median, 95% CI) to 1.82 (1.38-2.18) IU/ml (p=0.008), without significant change of the plasminogen activator antigen concentration (4.42 (2.50-7.81) to 3.91 (2.36-6.34), p=0.1954). This was associated by a substantial decrease in plasminogen activator inhibitor-1 activity from 10.4 (5.03-20.15) to 6.35 (4.11-10.75) IU/ml (p=0.0094). Furthermore, plasma levels of PGE_2 and TxB_2 markedly decreased (from 9.7 (5.00-15.22) to 6.70 (3.10-10.24) pg/ml (p=0.0048), and from 106.05 (39.1-266.73) to 70.20 (35.16-100.77) pg/ml (p=0.0023), respectively). During CsA, but not AZA, plasma tissue plasminogen activator-antigen and plasminogen activator inhibitor-1 levels correlated significantly with PGE_2 (r=0.53, p=0.02 and r=0.60, p=0.008, resp.) and TxB_2 (r=0.75, p=0.0001 and r=0.77, p=0.0001, resp.) levels.

In conclusion, CsA induced substantial impairment of fibrinolytic activity which recovered following conversion to azathioprine. The impaired fibrinolysis observed during CsA treatment may be caused by modulation of eicosanoid production or metabolism in vascular endothelial cells and possibly contributes to the high incidence of cardiovascular disease following kidney transplantation.

INTRODUCTION

In renal transplant recipients the morbidity and mortality due to atherosclerosis is substantially higher as compared to the normal population (1). Cardiovascular disease accounts for up to 40% of deaths after renal transplantation and there is increasing evidence that patients who are treated with cyclosporine A are at high risk (2-4). Hypertension and hyperlipidemia are undoubtedly involved, however, disturbances in the fibrinolytic system appear to play an important role in the pathogenesis of atherosclerosis and thrombosis as well (5,6). Impaired activity of the fibrinolytic system mostly results from excess plasminogen activator inhibitor-1 (PAI-1) and less commonly from reduced release of tissue- or urokinase- type plasminogen activator and has been associated with the use of the immunosuppressant cyclosporine A (CsA)(7). Due to endothelial cell damage and toxicity, CsA has been shown to increase the plasma levels of PAI-1, which results in decreased fibrinolytic activity (8). CsA-induced disturbances in prostanoid metabolism may also be implicated in the pathogenesis of the reduced fibrinolytic activity (9).

We therefore hypothesized that conversion from CsA to azathioprine (AZA) as long-term immunosuppressive therapy, would potentially ameliorate fibrinolytic activity. In the first prospective controlled study with paired addressing this issue, we compared several parameters of the fibrinolytic system in renal transplant patients during CsA and after conversion to AZA treatment, in order to determine the reversibility of the possible harmful effects on the fibrinolytic axis of long-term CsA. Plasma levels of prostaglandin E_2 (PGE_2) and thromboxane B_2 (TxB_2) were determined in order to assess whether CsA-induced interference with prostanoid metabolism is associated with impaired fibrinolysis, as has been suggested by others (9).

METHODS

Patients

Patients were recruited among renal transplant recipients who had been enrolled in a prospective randomized clinical trial, which was designed to evaluate the effects of two different immunosuppressive regimens, i.e. prednisone combined with CsA or with AZA, on long-term graft function and incidence of rejection episodes. In this trial all CsA-treated renal transplant recipients, between 18 and 65 years of age and 6 months or longer after

transplantation, were randomly allocated to either continuation of CsA treatment, or conversion from CsA- to AZA-based immunosuppression. Patients with diabetes mellitus, proteinuria of more than 3 grams per day, acute graft rejection in the previous three months, or histological evidence of chronic graft rejection were excluded. The first 18 consecutive patients fulfilling these criteria and who were willing to give written informed consent were included in the present study, which was approved by the Medical Ethics Committee of the University Hospital Rotterdam.

Study design

All patients were examined during CsA for the first time and for the second time during AZA. Both examinations were 16 weeks apart and the second examination took place 12 weeks after withdrawal of CsA. After the first examination, AZA was started at a dose of 2 mg/kg daily. Two weeks later the CsA dose was reduced to 50%, followed by discontinuation after another two weeks. The AZA dose was adjusted if anemia, thrombocytopenia, leucocytopenia occurred. Patients used the same dose of prednisone (range 7.5-12.5 mg daily) during both examinations. All antihypertensive medication (beta-blockers in 13 patients and calcium channel blockers in 8 patients) was discontinued at least 3 days prior to both study sessions.

Blood sampling and assays.

On both study days an intravenous catheter (Venflon, Viggo Spectramed, Helsingborg, Sweden) was inserted in the antecubital vein of the non-dominant arm. After 30 minutes of rest in supine position, venous blood samples were drawn in the appropriate anticoagulant. Platelet poor plasma was obtained by immediate centrifugation at 1600 x g for 20 minutes at 4°C. All serum and plasma samples were stored at -70°C until assayed.

Tissue plasminogen activator (tPA) activity was measured by an amidolytic assay (10). Briefly, 25 µl of plasma was mixed to a final volume of 250 µl with 0.1 M Tris- HCl, pH 7.5, 0.1% (v/v) Tween-80 and 0.3 mM S-2251 (Chromogenix, Mölndal, Sweden). The results are expressed as IU/ml. Plasminogen activator inhibitor-1 (PAI-1) activity was measured with an amidolytic assay (11), in which the samples were incubated with excess of tPA (40 IU/ml) for 10 min. at room temperature. The residual tPA activity was determined by incubation with 0.13 µM plasminogen (Chromogenix, Mölndal, Sweden), 0.12 mg/ml cyanogen bromide-digested fibrinogen fragments (tPA stimulator, Chromogenix, Mölndal,

Sweden) and 0.1 mM S-2251. The PAI-1 activity in the sample is inversely proportional to the plasmin generated in the mixture, determined by the conversion of the chromogenic substrate. Results are expressed in international units (IU), where 1 IU is the amount of PAI-1 that inhibits 1 IU t-PA. t-PA antigen and PAI-1 antigen were assayed with ELISA's (12)(Asserchrom t-PA, Diagnostica Stago, Asnieres-sur-Seine, France and PAI-1-ELISA kit, Monozyme, Charlottenlund, Denmark, respectively). All results are expressed in ng/ml. To assess plasmin generation *in vivo*, concentrations of plasmin complexed to α_2-antiplasmin (PAP complexes) were measured by a specific RIA (13). Briefly, specific monoclonal antibodies, raised against inactivated and complexed α_2-antiplasmin were coupled to Sepharose beads and incubated with plasma. After washing the Sepharose with phosphate-buffered saline, bound complexes were subsequently incubated with ^{125}I-labeled monoclonal antibodies against plasmin. After another washing step, Sepharose-bound radioactivity was measured. As standards, serial dilutions of plasma in which a maximal amount of PAP complexes was generated by incubation with two chain urokinase (Choay, Paris, France) were used. The results are expressed as nmol/l.
Prostaglandin E_2 and thromboxane B_2 were measured with competitive enzyme immunoassays (Cayman Chemical, Ann Arbor, MI, USA), using a monoclonal antibody against prostaglandin E_2 and specific polyclonal anti-thromboxane B_2 antibodies, respectively (14). Blood 12-hour trough CsA levels were determined with a polyclonal immunoassay (CycloTrac SP, Incstar, Stillwater, MN, USA).

Statistics

All data are presented as mean ± standard deviation for normally distributed data and as median and 95% confidence interval for data that were not normally distributed. Student's t-test and Wilcoxon's paired rank test were used for assessing the statistical significance of the changes after conversion from CsA to AZA. The relationships between different parameters were calculated with Pearson's correlation test.

RESULTS

Clinical characteristics

During CsA, mean arterial pressure was significantly higher (116 ± 15 mmHg vs. 106 ± 13 mmHg, p=0.0003) and serum creatinine was significantly higher (147 ± 34 μmol/l vs. 127 ±

35 μmol/l, p=0.002), than during AZA. Body mass index did not change upon conversion. The daily dose of prednisone was identical during both treatments (Table 1). No acute rejection episodes occurred between both examination sessions.

	CsA	AZA
Sex (male / female)	16 / 3	
Age (years)	41 ± 23	
Time after transplantation (mths)	24 ± 5	27 ± 5
Body mass index (kg/m^2)	24.9 ± 3.5	24.9 ± 3.3
Prednisone dose (mg/day)	9.6 ± 2.1	9.6 ± 2.1
CsA dose (mg/kg/day)	5.5 ± 1.7	-
AZA dose (mg/kg/day)	-	1.8 ± 0.4

Table 1. Patient characteristics

Fibrinolysis and prostanoids

Conversion from CsA to AZA was followed by an increase in tPA activity by 46 ± 71% (mean ± SD)(Figure 1).The plasma level of tPA activity during CsA treatment was 1.22 (0.84 -1.95)(median, 95% CI) IU/ml and during AZA 1.82 (1.38-2.18) IU/ml (p=0.0077)(normal value 1.72 (1.1-2.3) IU/ml). As shown in Figure 1, the increase in tPA activity appeared to be due to a reduction in PAI-1, as reflected by a decrease in plasma levels of PAI-1 antigen and associated activity, while the levels of tPA antigen did not change significantly (Table 2). The slight decrease in tPA antigen levels can be readily explained by the fact that this assay also measures tPA - PAI-1-complexes and will therefore be somewhat affected by decreasing PAI-1 levels. Median plasma PAI-1 activity and antigen levels decreased from 10.40 (5.0-20.1) to 6.4 (4.1-10.8) IU/ml (p=0.009)(normal value 7.7 (4.5-10.9) IU/ml) and from 13.4 (5.7-21.9) to 9.37 (5.6-13.1) ng/ml (p=0.04)(normal value 7.3 (4.5-10.1) ng/ml), respectively. The enhancement of tPA activity resulted in an increase of plasmin formation, as reflected by an increase in plasmin-α2-antiplasmin complex levels from 3.4 (1.8-7.0) to 5.2 (3.1-7.4), p=0.05 (Table

2). Plasma concentrations of both PGE_2 (-36 ± 108%) and TxB_2 (-43 ± 54%)(mean±SD) were lower during AZA treatment (Table 2).

	CsA	AZA	p
tPA-Ag (ng/ml)	4.4 (2.5-7.8)	3.9 (2.4-6.3)	0.19
PAP (nmol/l)	3.4 (1.8-7.1)	5.2 (3.1-7.4)	0.05
PGE_2 (pg/ml)	9.7 (5.0-15.2)	6.7 (3.2-10.2)	0.005
TxB_2 (pg/ml)	106.7 (39.1-266.7)	70.2 (35.2-100.8)	0.002

Table 2. Plasma concentrations of parameters of fibrinolysis and prostanoids during CsA and AZA tPA-Ag: tissue plasminogen activator antigen concentration; PAP: plasmin-α_2-antiplasmin complex concentration; PGE_2: prostaglandin E_2 concentration; TxB_2: tromboxane B_2 concentration. values are median (95% CI)

Correlation between prostanoids and fibrinolysis parameters.

During CsA, but not during AZA treatment, we observed strong positive correlations between plasma levels of PGE_2 and PAI-1-Ag (r=0.60, p=0.008)(Fig.2, left upper panel)

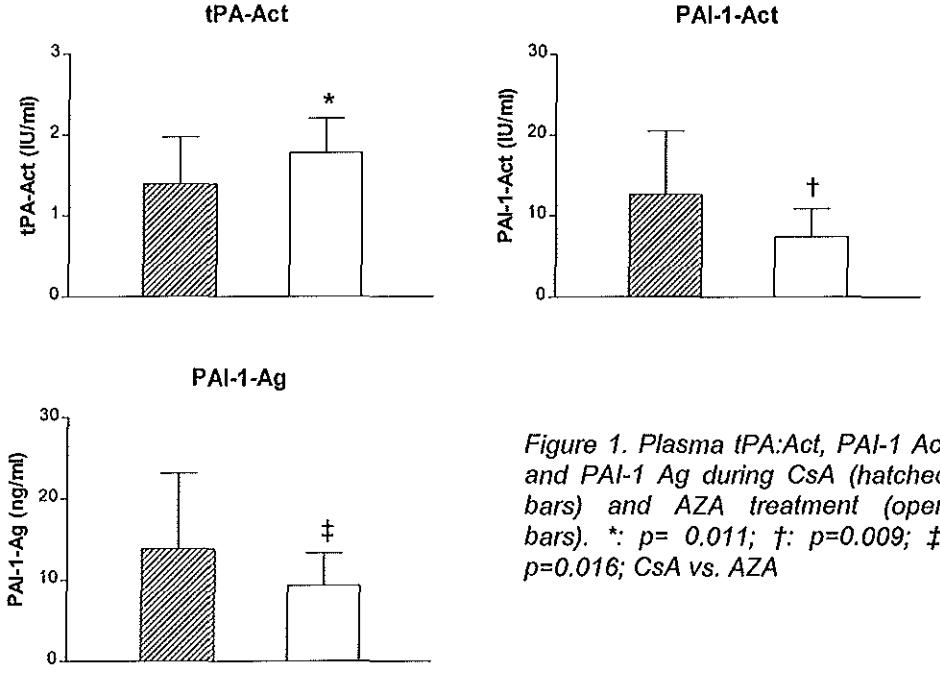

*Figure 1. Plasma tPA:Act, PAI-1 Act and PAI-1 Ag during CsA (hatched bars) and AZA treatment (open bars). *: p= 0.011; †: p=0.009; ‡: p=0.016; CsA vs. AZA*

and also plasma tPA-Ag (r=0.53, p=0.02). In addition, plasma TxB_2 was positively correlated to PAI-1-Ag (r=0.77, p=0.0001)(Fig 2, upper right panel) and tPA-Ag (r=0.75, p=0.0001). Interestingly, TxB_2 was negatively correlated to tPA activity (r=-0.70, p=0.001) (Fig 2, lower left panel), which suggests that TxB_2-induced elevation of PAI-1 inhibits tPA-Ag. This suggestion was supported by the negative correlation between TxB_2 and PAP-complex levels (r=-0.64, p=0.004)(Fig 2, lower right panel).

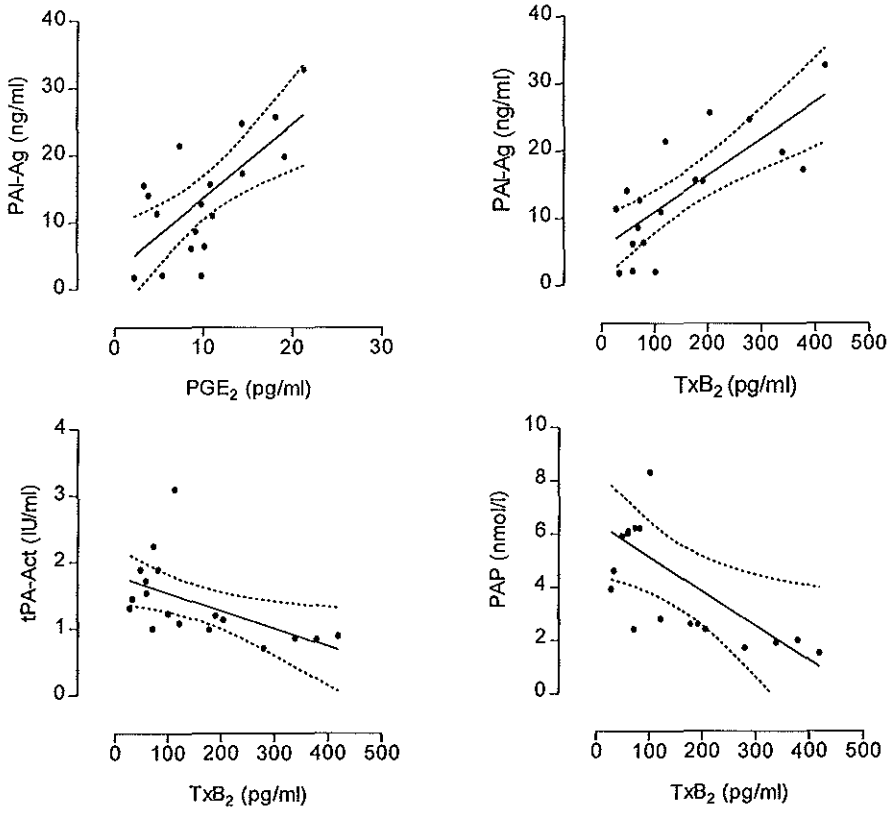

Figure 2.
Scatterplots and linear regression lines with 95% confidence interval of plasma PGE_2 and TxB_2, and some components of the fibrinolytic cascade.

DISCUSSION

The main finding emerging from our study is that in renal transplant recipients, the activity of the fibrinolytic system increases substantially after conversion from CsA to AZA. The reason for this improvement appears to be a decrease in plasma PAI-1 levels. During

CsA, but not AZA, we also found strong correlations between plasma prostanoid levels and several components of the fibrinolytic system.

In a number of studies, impaired fibrinolysis has been associated with the development of acute atherothrombotic events (5,6). We found that PAI-1 activity was significantly higher during CsA, which resulted in a reduction of tPA activity and subsequent plasmin formation as reflected by decreased plasmin-α_2-antiplasmin complexes. This finding is in agreement with data from a cross-sectional study, showing that in a group of CsA-treated renal transplant recipients PAI-1 was higher than in AZA-treated transplant recipients (15).

The mechanism of this CsA-associated impairment of fibrinolysis is not clarified. A defective release of tPA and PAI-1 upon DDAVP stimulation was reported in CsA treated renal transplant recipients, although basal tPA and PAI-1 levels were similar to those of AZA-treated patients (16). The impaired DDAVP-induced release of tPA was restored byfish-oil administration, suggesting that a CsA-induced alteration in prostanoid metabolism may be related to the impairment of fibrinolysis.

This hypothesis is supported by our finding that plasma PGE_2 and TxB_2 decreased markedly after conversion. This is in accordance with other data showing that during CsA systemic and intrarenal prostaglandin synthesis is disturbed (17,18). In addition, it has been shown that changes in eicosanoid dynamics may significantly affect vessel wall related fibrinolytic activity (9). The notion that alterations in prostanoid metabolism are involved in the attenuation of fibrinolytic activity during CsA, is further corroborated by our finding that plasma PGE_2 was closely correlated to plasma tPA-Act, PAI-1-antigen and -activity levels. In addition, TxB_2 was strongly negatively correlated to tPA-Act and PAP-complexes and positively correlated to PAI-1-Ag and PAI-1-Act. However, during AZA, no correlation between prostanoids and fibrinolytic parameters was present, which may suggest that after conversion the normal regulatory dynamics of fibrinolysis and prostaglandin synthesis was restored.

It is tempting to speculate about alternative explanations for the impairment of fibrinolysis during CsA. *In vitro*, oxidized LDL is a potent stimulator of the release of PAI-1 by cultured endothelial cells (19). Interestingly, we and others recently found that during CsA treatment, LDL is more oxidized, due to increased susceptibility to oxidation both *in vivo* and *in vitro* (20,21). Therefore, it is conceivable that oxidized LDL may enhance the release of PAI-1 *in vivo*, resulting in reduced fibrinolytic activity.

The decrease in PAI-1 activity may also be associated with the simultaneous fall in blood pressure following conversion (22). In a cross-sectional study of healthy young men, the incidence of hypofibrinolysis due to increased PAI-1 activity was increased in hypertensive subjects (23). The association between hypertension and impairment of fibrinolysis may be mediated by a third factor, e.g. hyperinsulinemia or insuline resistance (24). Other studies have demonstrated significant correlations between hypertriglyceridemia (which was also present during CsA), increased body weight and hypofibrinolysis due to increased PAI-1 (25).

Finally, an alternative mechanism may proceed via the LDL-receptor-related protein (LRP)/α_2-macroglobulin receptor, which is involved in the internalization and intracellular degradation of complexes of plasminogen activator and its inhibitor PAI-1 (26). CsA may lead to functional impairment of this receptor, leading to decreased PAI-1 clearance and consequently higher PAI-1 plasma levels.

In summary, we found that in renal transplant recipients, conversion from CsA to AZA is accompanied by a substantial amelioration of fibrinolytic activity. Our findings may contribute to the pathogenetic basis for the high incidence of cardiovascular disease after organ transplantation, which has been associated with the long term use of CsA (2-4). Whether or not the positive effects of long term treatment with CsA on graft survival are outweighted by the negative effects on cardiovascular morbidity and mortality, should be a topic for future studies.

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Chapter 7

FOREARM VASORELAXATION IN HYPERTENSIVE RENAL TRANSPLANT PATIENTS. THE IMPACT OF WITHDRAWAL OF CYCLOSPORINE

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J Hypertens, in press

ABSTRACT

Objective: To study whether cyclosporine A (CsA)-induced hypertension in renal transplant recipients is accompanied by impairment of endothelium-dependent vasodilatation, which has been suggested by *in vitro* and *in vivo* animal experiments.

Design and methods: *In vivo* endothelium-dependent and endothelium-independent vasodilatation and plasma concentrations of vasoactive hormones were determined in 16 renal transplant patients, while they were treated with CsA and 16 weeks later, after they were converted to azathioprine (AZA) therapy. The vasodilator response of the forearm vascular bed was measured by strain gauge venous occlusion plethysmography during intra-arterial infusion of acetylcholine (endothelium-dependent vasodilatation) and nitroprusside (endothelium-independent vasodilatation). Post-ischaemic reactive flow was measured after 10 minutes of arterial occlusion. In addition, plasma concentrations of noradrenaline and the prostanoids prostaglandin E_2 and thromboxane B_2 and also blood CsA concentration, were measured. Glomerular filtration rate and renal blood flow were estimated 1 day before the plethysmography study during each treatment period.

Results: Upon conversion from CsA to AZA mean arterial pressure fell significantly by 12 ± 3 % ($p<0.05$). Glomerular filtration rate and renal blood flow increased by 13 ± 5% and 19 ± 8% respectively (both $p<0.05$), while renal vascular resistance fell by 48 ± 11% ($p<0.01$). Both baseline forearm blood flow and baseline forearm resistance did not change after conversion (5.7 ± 0.7 vs. 4.9 ± 0.6 ml/100ml/min and 27.3 ± 4.2 vs. 26.2 ± 3.2 AU). The absolute and relative forearm blood flow response and forearm vascular resistance responses to acetylcholine (ACh) and nitroprusside (SNP) infusion were similar during CsA and AZA. Peak postischaemic forearm blood flow was 42 ± 12 % higher during CsA than during AZA ($p<0.05$), but the minimal post-ischaemic forearm vascular resistance between both treatments did not differ. Plasma PGE_2 and TxB_2 levels decreased by 34 ± 7% and 45 ± 8% after conversion, but noradrenaline levels did not change.

Conclusions: Our data indicate that, in renal transplant recipients, CsA-induced hypertension is not accompanied by an increased forearm vascular resistance. In addition, conversion from CsA to AZA was not followed by changes in endothelial vasodilator function, although mean arterial pressure decreased significantly. Our study does not

support the hypothesis that attenuation of endothelial vasodilator function contributes to the development of CsA-induced hypertension.

INTRODUCTION

After the introduction of the immunosuppressant cyclosporine A (CsA) in the transplantation field, graft survival rates have improved substantially (1). Unfortunately, the development of hypertension is a common side-effect of CsA, occurring in approximately 60 % of renal transplant recipients treated with CsA (2). Hemodynamically, CsA-induced hypertension is characterized by increased peripheral vascular resistance and a normal cardiac output (3). The etiology of CsA-induced hypertension is still unclear. Increased sympathetic nerve activity, an increase in intracellular free calcium concentration, enhanced release of endothelin-1, alterations in the metabolism of vasoactive prostaglandins and impaired endothelial vasodilator function have all been proposed to explain CsA-induced hypertension (reviewed in 4). The endothelium plays a crucial role in the regulation of vascular tone by the release of several vasoconstrictors, such as endothelin-1 and thromboxane A_2 and vasodilators, such as nitric oxide and prostaglandins E_2 and I_2 (5). Endothelium-dependent vasodilatation has been extensively studied *in vivo* in the forearm model and was found to be impaired in patients with diabetes mellitus, essential hypertension, hypercholesterolemia and congestive heart failure (6-9).

A number of *in vitro* and animal studies has provided evidence for impaired endothelium-dependent vasodilatation as a cause of CsA-induced vasoconstriction. Attenuation of endothelium-dependent vasodilatation of rat aorta segments and human skin arterioles was found after short-term incubation with very high CsA concentrations (10-12). In an *ex vivo* model of arteries of CsA exposed rats, CsA caused a marked decrease in endothelium-dependent vasodilatation and augmented noradrenaline-induced vasoconstriction (13). In intact rats, CsA inhibited endothelium-dependent renal vasodilatation (14). In CsA-treated rabbits, the renal vasodilator response to ACh was reduced as compared with untreated animals (15).

Other studies were unable to reproduce these findings (16), but instead found that CsA increased the sensitivity of vascular smooth muscle cells for vasoconstrictor agents, possibly by increasing transmembrane calcium flux and intracellular calcium concentration

(17-19). Studies in dogs have shown that intra-arterially administered CsA has vasoconstrictive effects, which appeared to be mediated by an increase of sympathetic tone caused by inhibition of noradrenaline re-uptake (20). In humans, treatment with CsA impaired venodilatation of dorsal hand veins induced by PGE_1 and isoproterenol, without increasing sympathetic activity (21).

In the present study we assessed whether in renal transplant patients with CsA-induced hypertension, endothelium-dependent vasodilatation, as a contributing factor in the pathogenesis of their hypertension, is impaired.

METHODS

Subjects

Sixteen renal transplant recipients who had developed hypertension (diastolic arterial pressure > 95 mmHg) after transplantation, were included in the study. These patients were recruited from a prospective randomized clinical trial, in which continuation of CsA was compared with conversion from CsA to AZA, with regard to long-term graft function and graft survival. Patients with posttransplant hypertension, who had been randomized for conversion from CsA to AZA were recruited to take part in the present study. All antihypertensive medication was discontinued at least 72 h prior to the studies. Only patients in whom the discontinuation of antihypertensive treatment was considered to carry no high risk for acceleration of hypertension, were included in the study. Patients with diabetes mellitus, peripheral vascular disease, Raynaud's phenomenon, hypercholesterolemia and smokers, were not eligible. All 16 patients were examined on two occasions, once while on CsA therapy and once after conversion to AZA, 16 weeks after CsA had been withdrawn. All participants gave written informed consent for the procedures. The protocol was approved by the Medical Ethics Committee of the University Hospital Rotterdam.

Conversion protocol

On the day after the first study AZA, at a dose of 1.5-2.0 mg/kg once daily, was added to the maintenance CsA and prednisone dose. Two weeks later, the CsA dose was reduced to 50% and the prednisone dose was increased to 30 mg daily. After another 2 weeks CsA was withdrawn, followed by stepwise dose reductions of prednisone till the baseline dose was reached 8 weeks later.

Experimental protocol

The studies were performed in the afternoon in a quiet room with an ambient temperature of 24 ^{0}C (22). The patients refrained from smoking and caffeine containing beverages for at least 24 h before the study.

After local anesthesia, a 1.1 mm diameter catheter (QuickCath, Baxter Healthcare, Thetford, Norfolk, UK) was inserted in the brachial artery of the nondominant arm. In patients with previous hemodialysis access surgery (n=12), the dominant arm was used. In all patients the same arm was used during both plethysmographic sessions and because of previous access surgery in the majority of patients plethysmographic measurements were performed unilaterally. The arm was supported 10 cm above the level of the right atrium. A mercury-filled Silastic strain gauge was positioned around the widest part of the forearm. The strain gauge was connected to a plethysmograph (model Periflow SU 4, Janssen Scientific Instruments, Beerse, Belgium) with electronic calibration for percentual volume changes and an inbuilt flow integrator module. To enable off-line data analysis, the plethysmograph was connected to an A/D converter (Dataq Instruments, model DI 420, Akron, OH, USA) for electronic data storage. For each venous occlusion cycle, a cuff placed around the upper arm was inflated to 50 mmHg with an ECG-triggered rapid cuff inflator (Janssen Scientific Instruments) during 6 heart beats, followed by deflation during 6 heart beats. A wrist cuff was inflated to 200 mmHg during the measurements to exclude circulation of the hand.

After a 30 minute rest period, baseline blood flow was measured for 5 min. during infusion of saline at a rate of 1 ml/min. Thereafter, forearm blood flow was measured during the infusion into the brachial artery of acetylcholine (ACh) at dosages of 1.0, 2.0, 4.0, 20.0 and 40.0 µg per minute, and sodium nitroprusside (SNP) at dosages of 1.0, 2.0, 4.0, 10.0, 20.0 and 40.0 µg per minute, for 4 minutes per dosage step. Both drugs were dissolved in saline and the infusion rates ranged from 0.5 to 2.0 ml per minute. Flow values obtained during the last minute of each dosage step were averaged and used for analysis. Between the infusion of ACh and SNP a 30 minute rest period was taken. Intra-arterial blood pressure, recorded immediately after each dosage step, was used for further calculations.

At the end of the intra-arterial infusion studies, the arterial forearm blood flow was interrupted by inflation of the cuff of the plethysmograph to 300 mmHg for 10 minutes.

Dynamic exercise (30 hand contractions) was added during the last minute. Immediately after cuff deflation, peak reactive hyperaemic forearm blood flow was recorded.

Before the plethysmographic measurements were performed, blood samples were drawn from the brachial artery for determination of CsA blood concentration and the plasma concentration of noradrenaline, plasma lipid profile and prostaglandin E_2 (PGE_2) and thromboxane B_2 (TxB_2). The plasma concentration of noradrenaline was determined by fluorimetric detection after HPLC separation (23). Plasma total cholesterol, LDL-cholesterol, HDL-cholesterol and triglyceride concentration were measured as described previously (24).

Plasma PGE_2 and TxB_2 concentrations were measured with a commercially available competitive enzyme immunoassay using a monoclonal anti-PGE_2 antibody and polyclonal anti-TxB_2 antibodies (Cayman Chemical, Ann Arbor, MI, USA). CsA blood levels were determined with a polyclonal assay (CycloTrac SP, Incstar, Stillwater, MN, USA). On the day before the forearm studies glomerular filtration rate (GFR) and renal plasma flow were estimated from the clearance of ^{125}I-thalamate and ^{131}I-hippurate (25).

Statistics

Forearm blood flow is expressed as ml per minute per 100 ml forearm volume. Forearm vascular resistance (FVR) is calculated as the mean arterial pressure (MAP) divided by forearm blood flow (FBF) and given in arbitrary units (AU). To correct for differences in basal forearm blood flow and vascular resistance, the responses are expressed as the percentage of baseline values and as absolute change from baseline values. Of each individual patient, the dose of ACh and SNP causing an increase of 100% in FBF and the dose causing 50% reduction in FVR, were calculated from a linear regression curve of the observed individual log[dose)-response relationship. Renal blood flow (RBF) was calculated by dividing effective renal plasma flow by (1-hematocrit). Renal vascular resistance (RVR) was calculated by dividing MAP by RBF. GFR, RBF and RVR are given as values per 1.73 m^2 body surface area. To compare mean values, Student's t-test for paired or unpaired data was used. The responses of patients to infusion of ACh and SNP, during CsA treatment and after conversion to AZA, were compared by analysis of variance for repeated measures. All p-values are two-tailed and the level of significance is set at a p value of 0.05. All values are means ± SEM, unless otherwise indicated.

RESULTS

Patient characteristics

Characteristics of the patients are shown in Table 1. After conversion from CsA to AZA, mean intra-arterial pressure fell from 115 ± 4 mmHg to 106 ± 4 mmHg ($p<0.01$)

	CsA	AZA	p
Number of patients	16		
Sex M / F	14/2		
Age (yrs)	41 ± 3		
Weight (kg)	76 ± 4	76 ± 4	n.s.
Period post transplantation (months)	24 ± 5	-	
CsA dose (mg/kg/day)	5.5 ± 0.4	-	
CsA 12-h trough level (ng/ml)	250 ± 16	-	
CsA level during plethysmography (ng/mL)	534 ± 14	-	
Prednisone dose (mg/day)	9.6 ± 0.3	9.6 ± 0.3	n.s.
Azathioprine dose (mg/kg/day)	-	1.8 ± 0.1	
Number of antihypetensives / patient	1.3 ± 0.3	0.3 ± 0.2	<0.05

Table 1. Clinical characteristics of patients during CsA and AZA treatment.

Resting heart rate was similar during CsA and AZA: 84.7 ± 3.7 vs. 87.7 ± 4.0 beats per minute. During CsA, 6 patients were treated with calcium antagonists only, 4 with beta blockers only, 5 with the combination of both categories, and 1 patient was without antihypertensive therapy. After conversion, 2 patients were treated with calcium antagonists and 3 with beta blockers. The number of antihypertensive drugs per patient decreased from 1.3 ± 0.3 to 0.3 ± 0.2 (Table 1). After conversion, GFR and RBF increased significantly, accompanied by a marked decrease in RVR (Table 2). As expected, CsA blood levels during the forearm studies were higher than the 12-hour trough blood levels (Table 1). Plasma total cholesterol, LDL-cholesterol and triglyceride concentrations fell significantly after conversion, while HDL-cholesterol remained stable (Table 3).

Forearm vascular response

After conversion baseline FBF and FVR did not change (Table 2). The absolute and relative increase in FBF and the relative decrease in FVR upon infusion of ACh and SNP were similar during both treatments (Fig 1 and 2). The calculated doses of ACh and SNP

	CsA	AZA	p
Basal forearm blood flow (mL /100 mL/min)	5.7 ± 0.7	4.9 ± 0.6	n.s
Basal forearm vascular resistance (AU)	27.3 ± 4.2	26.2 ± 3.2	n.s.
Glomerular filtration rate (mL/min)	50 ± 3	57 ± 5	<0.05
Renal blood flow (mL/min)	356 ± 33	436 ± 41	<0.05
Renal vascular resistance (mmHg.mL.min^{-1})	0.46 ± 0.05	0.37 ± 0.06	<0.01

Table 2. Basal forearm and renal hemodynamics during CsA and AZA.

leading to a 100% increase of FBF, or to a 50% reduction of FVR were not significantly different during CsA and AZA (Table 4). None of the parameters of forearm vascular responsiveness was different between patients treated with calcium entry blockers or patients treated with other antihypertensive agents.

	CsA	AZA	p
Total cholesterol (mmol/l)	5.8 ± 0.9	4.9 ± 0.7	<0.005
LDL-cholesterol (mmol/l)	3.7 ± 0.7	3.0 ± 0.6	<0.05
HDL-cholesterol (mmol/l)	1.1 ± 0.4	1.2 ± 0.4	n.s.
Total triglyceride (mmol/l)	2.6 ± 1.0	1.8 ± 0.6	<0.001

Table 3. Plasma lipid concentrations before and after conversion from CsA to AZA.

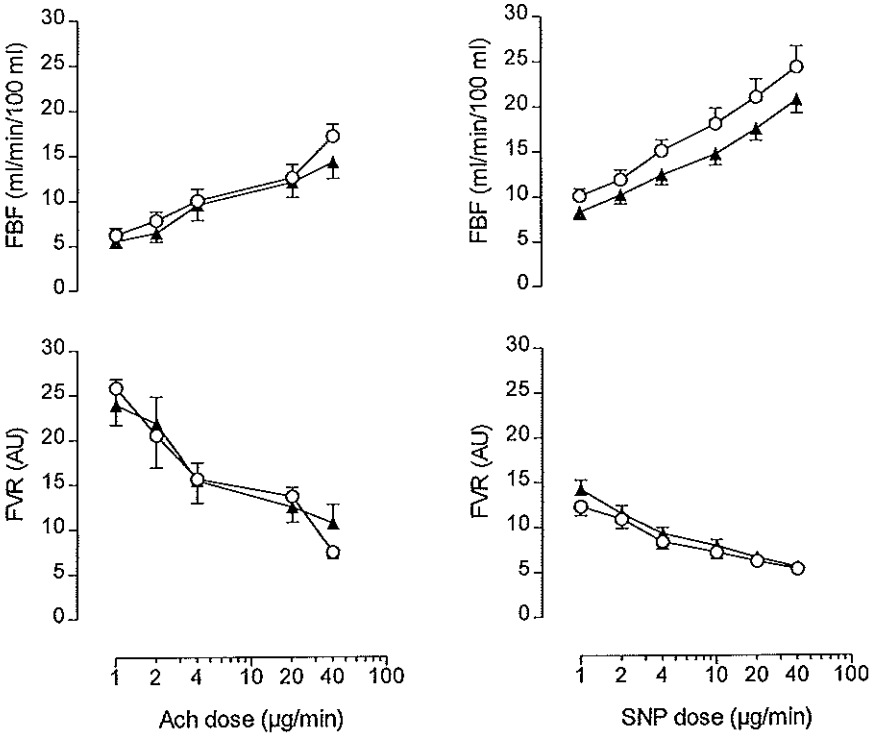

Figure 1. Absolute increase from baseline in forearm blood flow (FBF) during intra-arterial infusion of incremental doses of acetylcholine (ACh)(upper left panel) and sodium nitroprusside (SNP)(lower left panel), and absolute decrease from baseline in forearm vascular resistance (FVR) during intra-arterial infusion of incremental doses of acetylcholine (ACh)(upper right panel) and sodium nitroprusside (SNP)(lower right panel), during CsA (circles) and AZA (triangles). Mean ± SEM.

Postocclusion forearm flow

Postocclusion blood flow was measured in 13 out of 16 patients, in 3 patients the full occlusion period could not be completed because of discomfort. The peak forearm blood flow after deflation of the upper arm cuff decreased from 43.7 ± 5.9 ml/100 ml/min.

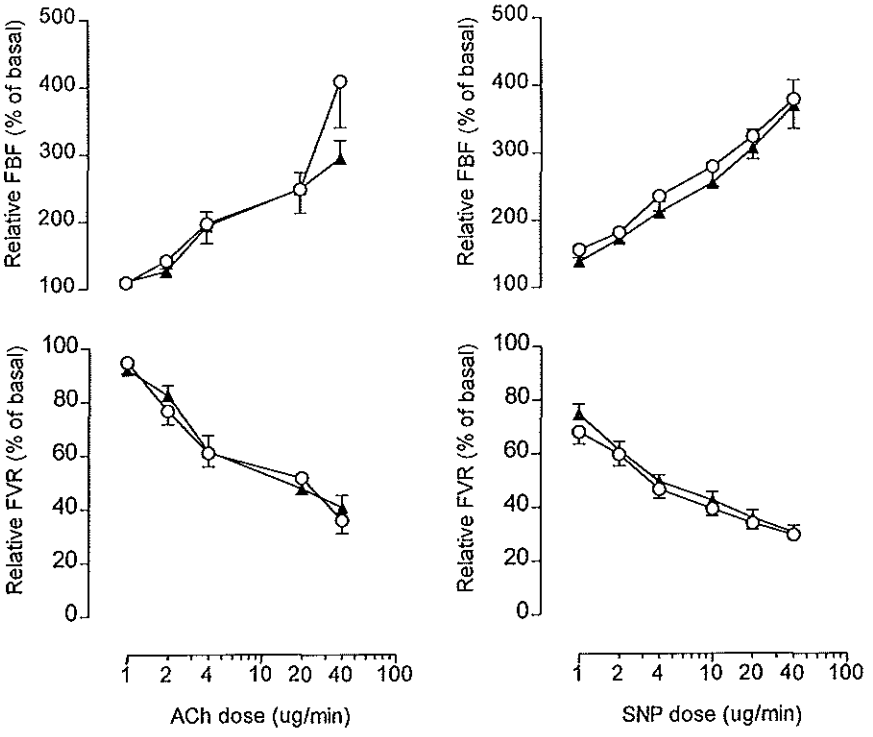

Figure 2. Relative increase from baseline in forearm blood flow (FBF) during intra-arterial infusion of incremental doses of acetylcholine (ACh)(upper left panel) and sodium nitroprusside (SNP)(lower left panel), and relative decrease from baseline in forearm vascular resistance (FVR) during intra-arterial infusion of incremental doses of acetylcholine (ACh)(upper right panel) and sodium nitroprusside (SNP)(lower right panel), during CsA (circles) and AZA (triangles). Mean ± SEM.

(median 40.6, range 16.2 to 74.9) during CsA, to 29.0 ± 3.6 ml /100 ml/min. (median 26.6, range 16.4 to 54.3) during AZA ($p<0.05$). However, the minimal postischemic forearm vascular resistance was similar: 3.0 ± 0.4 vs. 3.8 ± 0.4 AU (CsA vs. AZA resp., n.s.).

	CsA	AZA	p
Acetylcholine (μg/min)			
Forearm blood flow			
200% of baseline	7.5 (3.0-26.7)	5.1 (1.9-20.5)	n.s.
Forearm vascular resistance			
50% of baseline	19.8 (8.0-30.3)	13.8 (3.9-42.8)	n.s.
Sodium nitroprusside (μg/min)			
Forearm blood flow			
200% of baseline	2.8 (1.1-5.8)	4.4 (1.6-8.3)	n.s.
Forearm vascular resistance			
50% of baseline	3.2 (1.9-5.6)	3.8 (2.6-7.2)	n.s.

Table 4. Mean doses of acetylcholine and sodiumnitroprusside (95%CI) resulting in a 100% increase of forearm blood flow, or a 50% reduction in forearm vascular resistance during CsA and AZA.

Catecholamines and prostanoids.

The arterial plasma concentration of noradrenaline was 288 ± 31 pg/ml during CsA and 300 ± 37 pg/ml during AZA (n.s.). The plasma levels of prostanoids decreased substantially upon conversion: PGE_2 fell from 10.1 ± 1.3 to 6.3 ± 0.9 pg/ml ($p<0.001$), and TxB_2 fell from 157.3 ± 21.7 to 69.2 ± 9.2 ($p<0.005$).

DISCUSSION

In this study, we assessed whether CsA-induced hypertension in renal transplant patients is accompanied with impairment of forearm endothelial vasodilator function. For this purpose, patients with CsA-induced hypertension, who were converted to AZA-based immunosuppressive treatment, were studied.

Conversion from CsA to AZA markedly decreased arterial pressure, indicating that our study population was indeed appropriate to examine the effects of CsA on *in vivo*

human endothelial vasodilator function. The observed decrease in arterial pressure was not accompanied by a fall in baseline forearm vascular resistance, nor by improvement of endothelium-dependent vasodilatation.

Our findings are at variance with the results of several *in vitro* and animal studies, in which a decrease of endothelium-dependent vasodilatation after administration of CsA could be demonstrated (10-12). However, they are in agreement with the finding that CsA did not affect *in vivo* endothelial function in humans, as reflected by intact endothelial release of nitric oxide by human coronary arteries during CsA treatment (25). Differences in the CsA blood concentration and duration of CsA exposure, and interspecies differences in sensitivity to the effects of CsA may account for this discrepancy. In this regard, especially the duration of exposure to CsA may be important.

It has been demonstrated that the gene expression of nitric oxide synthase III, as well as the release of nitric oxide in rat kidney and human endothelial cells is increased in response to short-term CsA administration (26, 27). This increased activity of the nitric oxide system, which might protect against CsA-induced vasoconstriction, is likely more enhanced after long-term than after short-term CsA exposure. Although we did not measure parameters of nitric oxide production, enhancement of nitric oxide production and release by CsA may explain why no effect on endothelium-dependent vasodilatation was detectable in the present study. The observation that the post-ischaemic peak forearm blood flow was higher during CsA than during AZA, also supports the possibility that an intrinsic vasodilator mechanism was activated during CsA therapy. Apparently, the compensation for the CsA-induced vasoconstriction by vasodilator mechanisms such as the nitric oxide pathway was insufficient to compensate for the systemic and renal vasoconstrictive effects of CsA, since arterial pressure and renal vascular resistance were considerably higher during CsA than during AZA.

Modification of the release of endothelium-dependent vasoactive substances has been proposed to play a role in the pathogenesis of CsA-associated hypertension (4). In the present study, we found that plasma levels of thromboxane A_2 and PGE_2 were both increased during CsA therapy. This confirms earlier reports of elevated plasma prostanoid levels during CsA (28). In addition, it has been shown that CsA impairs fibrinolytic activity by enhancement of the endothelial release of plasminogen activator inhibitor-1 (29).

There is evidence from clinical studies that CsA treatment is associated with an increase in the activity of the sympathetic nervous system, either through a central effect

or inhibition of noradrenaline re-uptake (20,30). Most likely, this sympathetic activation is very differentiated, since in our patients arterial plasma noradrenaline concentration, as an approximate index of overall sympathetic tone, was not higher during CsA than during AZA.

In the study participants, hypertension was treated predominantly with calcium channel blockers. Improvement of endothelial vasodilator function by these agents has been reported (31). During CsA the dose and number of antihypertensive agents was higher because of more severe hypertension. This may have masked CsA-induced endothelial dysfunction. In our opinion this contention is unlikely, in view of the rather short half-life of the antihypertensive drugs, which were taken two or three times daily and the withdrawal of all antihypertensives three days prior to the studies. In addition, the forearm vasodilator response was not associated with the severity of hypertension or the class of antihypertensive drugs that was used.

During CsA treatment, 11 patients used calcium entry blockers, whereas during AZA this number was reduced to two. Since there is some evidence that calcium entry blockers improve endothelium-dependent vasodilatation (31), it can not be ruled out that the potential negative effect of CsA on endothelium-dependent vasodilatation was to some extent masked by the use of this class of antihypertensive agents. We have attempted to avoid such an interaction by the discontiunation of all antihypertensive drugs 3 days before the studies. The vasodilator responses to Ach and SNP, both during CsA and AZA, were not different in patients who were treated with or without calcium entry blockers.

Since CsA is the first choice initial immunosuppressant after renal transplantation, patients were always switched from CsA to AZA and not the other way around. It can therefore not be completely ruled out that an order effect might have influenced our findings. Although baseline forearm blood flow might be sensitive to an order effect, this is hard to imagine for the vasodilator responses to intra-arterial infusion of ACh and SNP.
Our finding that endothelium-dependent vasodilatation was not impaired in renal transplant recipients with CsA-induced hypertension, suggests that the capacity of endothelial cells in the forearm to release vasodilators such as nitric oxide and vasodilator prostanoids was preserved during CsA. We conclude that impairment of endothelium-dependent vasodilatation does not play a key role in the pathogenesis of CsA-induced hypertension after renal transplantation.

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Chapter 8

SYSTEMIC AND RENAL HEMODYNAMIC EFFECTS OF L-ARGININE INFUSION IN RENAL TRANSPLANT RECIPIENTS. THE INFLUENCE OF CYCLOSPORINE WITHDRAWAL

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ABSTRACT

To investigate whether impairment of nitric oxide(NO)-induced vasodilatation contributes to the development of cyclosporine (CsA) hypertension and nephrotoxicity, we examined the effects of intravenous infusion of L-arginine (L-Arg), the substrate for the NO-producing enzyme NO-synthase, on systemic and renal hemodynamics in 16 renal transplant recipients. The same protocol was performed twice, before and after these patients were converted from CsA to azathioprine (AZA) treatment. During CsA, the fall in mean arterial pressure and increase in heart rate were more prominent than during AZA. During both treatments, the peak glomerular filtration rate and renal blood flow after L-Arg were comparable, despite lower baseline values during CsA. Consequently, the renal vascular resistance decreased more during CsA, while the nadir was similar during both treatments. Basal L-citrulline levels were higher during CsA, but the L-Arg-induced increase was not different. L-Arg infusion was followed by a comparable increase in plasma norepinephrine and a comparable decrease in plasma atrial natriuretic peptide during CsA and AZA. L-Arg infusion did not affect plasma renin during CsA or AZA. L-Arg induced a transient increase in urine flow, FE_{Na} and urinary anion gap, but no difference was observed between the two treatments.

We conclude that the L-Arg-induced systemic and renal hemodynamic responses are not essentially different during CsA and AZA, although the relative increase in glomerular filtration rate and renal blood flow was larger during CsA. L-Arg caused similar effects on vasoactive hormones during both treatments. Our findings do not support the proposition that impairment of endothelial NO production contributes to the pathogenesis of CsA-induced hypertension and nephropathy.

INTRODUCTION

CsA is the mainstay of immunosuppressive therapy after clinical transplantation (1) and is increasingly employed outside the transplantation field. However, the use of CsA is complicated by nephrotoxicity and hypertension in a substantial fraction of patients (2,3). CsA is a potent constrictor of renal afferent glomerular arterioles, leading to reduction of renal blood flow and glomerular filtration rate (2). In addition, there is evidence that CsA constricts systemic resistance vessels, which in combination with renal sodium retention, may, at least in part, explain the development of hypertension (4). The precise etiology of this vasoconstrictive action of CsA is yet unclear. Several pathogenetic factors may be

involved, like interference with intrarenal prostaglandin synthesis, leading to an imbalance between vasoconstrictor and vasodilator prostanoids, activation of the renin-angiotensin-aldosterone system, or the sympathetic nervous system and alterations in intracellular calcium handling (reviewed in 5). In addition, impairment of endothelial nitric oxide (NO)-mediated vasodilator function may be an important mechanism by which CsA affects systemic and renal vessel tone (6,7). The endothelium plays a key role in the regulation of regional vascular tone. Several *in vitro* and *in vivo* animal studies indicate that CsA interferes with the normal vasoregulatory function by reducing the production of NO by renal and systemic endothelial cells (7-9). Vascular endothelial cells synthesize NO from L-arginine (L-Arg) through the action of the intracellular enzyme NO-synthase and secrete NO in a paracrine fashion resulting in smooth muscle cell relaxation (10). Intravenous administration of L-Arg increases renal perfusion, which may be mediated by increased intrarenal NO production, although NO-independent mechanisms may be operative as well (11,12). The increased NO production possibly results from the supply of excess substrate for this enzyme and/or activation of the inducible isoform of NO-synthase (9,13). Apart from its renal vasodilatory effects, infusion of L-Arg in humans lowers blood pressure through peripheral vasodilatation, which may also result from increased endothelial NO production (14).

To assess the role of CsA on both systemic and renal NO-mediated vasodilator function in the pathogenesis of CsA-induced hypertension and nephrotoxicity, we administered L-Arg intravenously to renal transplant recipients, both during treatment with CsA and after they were converted from CsA to AZA treatment.

METHODS

Subjects

Sixteen renal transplant patients were recruited from the renal transplant outpatient clinic at the University Hospital Rotterdam. These patients participated in an ongoing prospective randomized trial to assess the effects of conversion from CsA to AZA on long-term graft function and graft survival. For this trial all patients of our clinic, who were at least 6 months after transplantation, were randomly allocated to either conversion to AZA or continuation of CsA treatment. Only non-diabetic patients who had a stable allograft function (serum creatinine < 200 μmol/l) were included. The first 16 consecutive patients meeting these criteria and willing to give informed consent, participated in the present

study protocol. All antihypertensive medication was discontinued at least three days before the studies.

Study protocol

The same study protocol was performed twice: while patients were treated with CsA and 16 weeks later, while the same patients were treated with AZA. On both study days patients arrived in the cardiovascular research laboratory after an overnight fast. At arrival they were weighted and subsequently a small catheter (Venflon, Viggo Spectramed, Helsingborg, Sweden) was inserted in a forearm vein of each arm. One catheter was used for the infusion of radio-labeled thalamate and hippuran for renal function studies and infusion of L-Arg, and the other catheter was used for blood sampling. On the day before the renal function studies patients collected urine for measurement of 24-h sodium excretion. Immediately after the first study, AZA was started at a dose of 2 mg/kg/day and CsA was gradually withdrawn. The protocol was approved by the Medical Ethics Committee of the University Hospital Rotterdam.

Measurements

Systemic hemodynamics

During the whole study, blood pressure and heart rate were recorded at 2.5-minute intervals, starting 1 h before the infusion of L-Arg, with an automated oscillometric device (AccuTorr3, Datascope, Montvale, NJ, USA). Ten-minute averages were used for further analysis.

Renal hemodynamics

Effective renal plasma flow (ERPF) and glomerular filtration rate (GFR) were estimated from the clearance of ^{131}I-hippuran and ^{125}I-thalamate (Amersham International Plc, Little Chalfont, Buckinghamshire, UK) respectively. After an iv. priming dose both radiopharmaceuticals were infused at a constant rate (15). Urine was collected at 30-minute intervals. To maintain sufficient urinary output, patients drank 15 ml/kg tap water 1 hour before the beginning of the infusions and the volume of each voided portion of urine was substituted orally with tap water. After an equilibration period of 90 minutes, 30-minute urine collections were used to measure the urine concentration of both radiopharmaceuticals, and blood samples were taken at 30-minute intervals to measure

their plasma concentration. Two hours after the beginning of the infusion of the radiopharmaceuticals, 300 ml L-Arg-HCl solution (concentration 100 mg L-Arg-HCl/ml; Bufa Chemie, Uitgeest, The Netherlands) was administered at a rate of 1000 mg per minute, for 30 minutes. The total L-Arg dose of 30 g represented a total acid and Cl load of ≈ 160 mmol each. Renal blood flow (RBF) was calculated by dividing effective renal plasma flow by (1- hematocrit). Renal vascular resistance (RVR) was calculated by dividing MAP by RBF. All data of GFR, RBF and RVR are reported as values per 1.73 m^2 body surface area.

Laboratory measurements

For measurement of plasma norepinephrine, blood was collected into chilled 10-ml heparinized polystyrene tubes containing 12 mg of glutathione, for measurement of plasma ANP and endothelin-1 in chilled 10-ml tubes containing EDTA (19 mg) and aprotinine (1000 kIU) and for measurement of plasma renin in chilled 10-ml tubes containing EDTA (19 mg). The tubes were centrifuged immediately after sampling (10 min, 3000 x g, 4°C), and the plasma was separated and stored at -80 °C until assayed. Plasma norepinephrine concentration was determined by HPLC with fluorimetric detection (16). Active plasma renin concentration was measured as previously described (17). Measurements of plasma ANP and endothelin-1 concentration were performed after SepPak C18 extraction, with commercially available radioimmunoassay kits from the Nichols Institute, Wijchen, The Netherlands (18,19). Plasma concentrations of L-arginine and L-citrulline were determined by HPLC with fluorimetric detection after derivatization with o-phtaldialdehyde, as described by van Eijk et al. (20). Prostaglandin E_2 and thromboxane B_2 were measured with commercially available competitive enzyme immunoassays (Cayman Chemical, Ann Arbor, MI, USA), using a monoclonal antibody against prostaglandin E_2 and specific polyclonal anti-thromboxane B_2 antibodies, respectively. Plasma and urine sodium, potassium and chloride were determined by flame photometry. Urine anion gap was calculated as $[Na^+ + K^+) - Cl^-$, and used as an approximate indicator for urinary acid excretion.

Before the start of the renal function studies a blood sample was taken for the determination of CsA 12-hour trough level in blood (CycloTrac SP, IncStar Corp., Stillwater, MN, USA).

Statistical analysis

For comparison of parameters during CsA and AZA treatment, the t-test for paired observations was used. For analysis of the influence of conversion from CsA to AZA on the effects of L-Arg infusion repeated measures ANOVA was used. All values are presented as mean ± SEM. The level of significance was set at a p value of 0.05.

RESULTS

Patient characteristics

Sixteen renal transplant recipients (11 males), age 40 ± 2 yr. (mean ± SD), weight 76 ± 15 kg, who were 24 ± 5 months after transplantation, participated in the study. None of the patients experienced a graft rejection episode between the two studies. The infusion of L-Arg was well tolerated by all subjects, and noticeable side effects did not occur. Infusion of L-Arg increased the plasma concentration of L-Arg about 40-fold; from 77 ± 81 to 2920 ± 2502 μmol/l during CsA and from 76 ± 73 to 3208 ± 2632 μmol/l during AZA).

	CsA	AZA	p
Mean arterial pressure (mmHg)	136.5 ± 18.0	121.2 ± 13.5	0.0006
No. of antihypertensive drugs (no/pt)	1.3 ± 0.3	0.3 ± 0.2	0.0001
Prednisone dose mg/day)	9.6 ± 0.3	9.6 ± 0.3	n.s.
CsA dose (mg/kg/day)	5.5 ± 0.4	-	
CsA 12-h trough level (ng/ml)	250 ± 16	-	
AZA dose (mg/kg/day)	-	1.8 ± 0.2	
Serum electrolytes			
Na (mmol/l)	140 ± 2	142 ± 2	n.s.
K (mmol/l)	4.4 ± 0.6	4.3 ± 0.5	n.s.
Cl (mmol/l)	105 ± 3	104 ± 4	n.s.
Urine Na excretion (mmol/day)	188 ± 108	187 ± 108	n.s.

Table 1. Clinical characteristics of the patients during CsA and AZA treatment at the time of the experimental studies; n.s.: not significant

Systemic hemodynamics

Baseline MAP was considerably higher during CsA than during AZA (Table 1), but resting HR was similar (77.9 ± 3.3 vs. 76.0 ± 2.9 bpm, n.s.). After infusion of L-Arg, MAP fell more during CsA than during AZA (8.9 ± 2.0% vs. 4.8 ± 1.4%, $p<0.05$). In addition, in response to L-Arg infusion HR increased significantly during CsA but not during AZA (Figure 1).

Renal hemodynamics

After conversion from CsA to AZA baseline GFR increased from 50 ± 3 to 57 ± 5 ml/min ($p<0.05$) and RBF increased from 337 ± 34 to 415 ± 40 ml/min ($p<0.01$) (Figure 2).

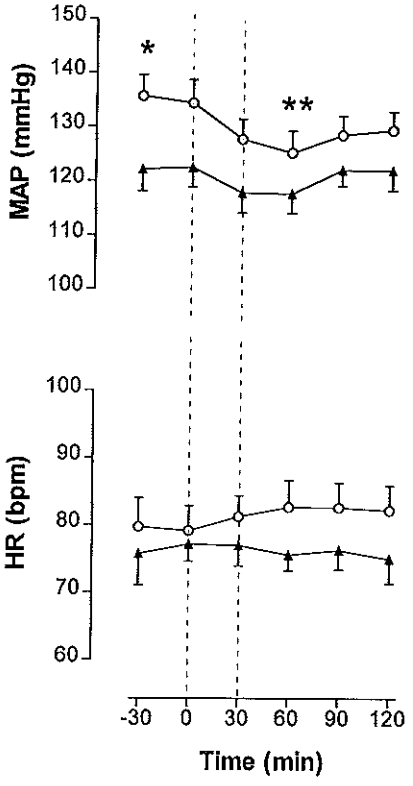

*Figure 1. Mean arterial pressure and heart rate before, during and after L-Arginine infusion during CsA (open circles) and AZA (closed triangles). L-Arginine was administered between the hatched lines. * $p<0.05$, CsA vs.AZA; ** $p<0.05$, vs.baseline*

L-Arg infusion was associated with an increase in GFR, which was more prominent during CsA than during AZA (16 ± 3 vs. 5 ± 3 %, $p<0.005$) (Figure 2). Likewise, the L-Arg-induced increase in RBF was considerably larger during CsA than during AZA (34 ± 6% vs. 7 ± 5%, $p<0.05$), leading to a comparable peak value of RBF during both treatments (Figure 2). In response to L-Arg infusion the decrease in RVR was substantially larger during CsA than during AZA (27 ± 3 vs. 5 ± 5%, $p<0.01$)(Figure 2), but during both treatments the nadirs of RVR were similar (Figure 2) and were reached at the end of the L-Arg infusion. After this initial decrease, RVR increased again, resulting in about the same RVR values at the end of the experiments (Figure 2). The filtration fraction did not change after conversion from CsA to AZA, or in response to L-Arg infusion.

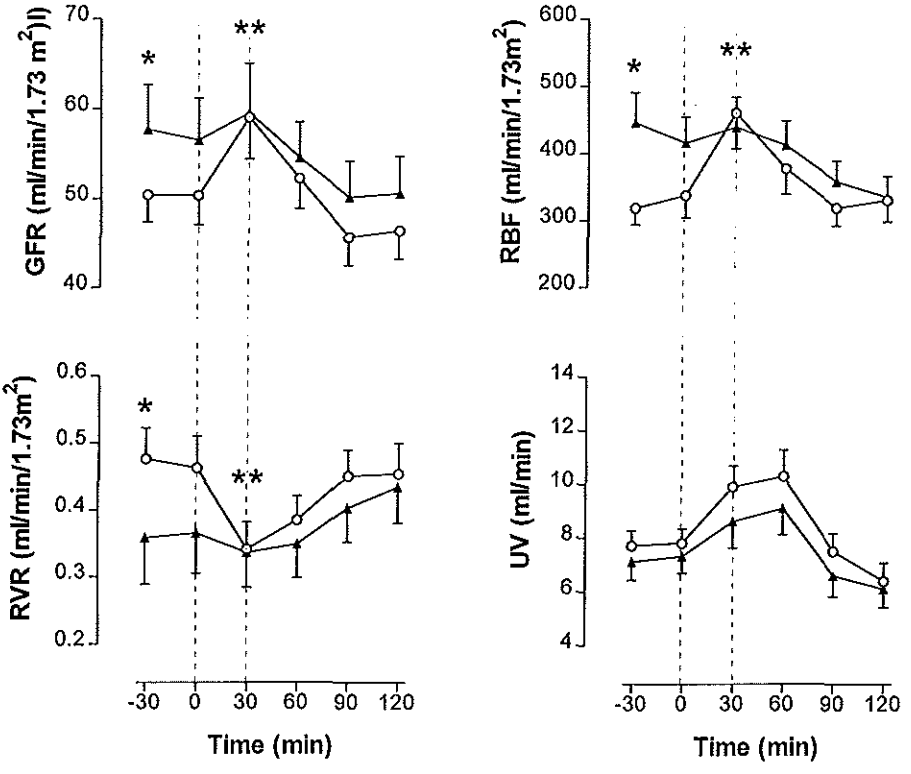

*Figure 2. Glomerular filtration rate, renal blood flow, renal vascular resistance and urine production, before, during and after L-Arginine infusion during CsA (open circles) and AZA (closed triangles). L-Arginine was administered between the hatched lines. * p<0.05, CsA vs.AZA; ** p<0.05, vs.baseline.*

Water and electrolyte excretion

Urine flow increased substantially in response to L-Arg both during CsA and AZA. After this initial rise urine production fell below the baseline level (Figure 2). Twenty four-hour urinary sodium excretion before both study sessions was almost identical (Table 1). Both baseline FE_{Na} and the increase in FE_{Na} in response to L-Arg administration were slightly higher during CsA than during AZA (Table 2). Urinary acid excretion increased considerably after the start of the L-Arg infusion, and had not returned to baseline after 120 minutes. The increase in urinary acid excretion in response to L-Arg infusion during CsA and AZA was comparable (Table 2).

	Before	During	After	p	
				CsA vs AZA	L-Arg vs baseline
UV (ml/min)					
CsA	7.8 ± 0.5	10.3 ± 1.0	6.4 ± 0.7	n.s.	<0.01
AZA	7.3 ± 0.7	9.1 ± 1.0	6.1 ± 0.7		<0.01
FE_{Na} (%)					
CsA	0.29 ± 0.12	0.48 ± 0.46	0.32 ± 0.31	n.s.	<0.01
AZA	0.21 ± 0.16	0.36 ± 0.25	0.27 ± 0.20		<0.01
U anion gap (μmol/min)					
CsA	77 ± 26	-203 ± 59	7 ± 21	n.s.	<0.001
AZA	142 ± 48	-237 ± 43	-18 ± 15		<0.001

Table 2. Water and electrolyte excretion before, during and after L-Arg infusion during CsA and AZA treatment; n.s.: not significant

Vasoactive hormones

The baseline plasma norepinephrine concentration during both treatments was similar, but the L-Arg induced increase in plasma norepinephrine was much more prominent during CsA than during AZA (Table 3). The plasma renin concentration was not affected by the conversion from CsA to AZA, or by the infusion of L-Arg. The baseline

plasma ANP concentration during CsA and AZA was similar. However, after an initial stable period, ANP levels fell significantly after the L-Arg infusion (Table 3). Although endothelin-1 levels were not changed by conversion itself, they increased slightly after L-Arg infusion during CsA (Table 3). After conversion, plasma PGE_2 concentration fell from 10.1 ± 1.3 to 6.7 ± 0.9 pg/ml (p=0.007) and plasma TxB_2 concentration fell from 153 ± 29 to 68 ± 8 pg/ml (p=0.003). The baseline plasma levels of L-citrulline were significantly higher during CsA than during AZA (41 ±14 vs. 34 ± 12 μmol/l, p=0.009), but the increase after the L-Arg infusion was comparable (absolute values 51 ± 14 vs. 44 ±14 μmol/l, p<0.05; mean increase 10 ± 6 vs. 11 ± 6 μmol/l, not significant).

	Before	During	After	p value	
				CsA vs AZA	L-Arg vs baseline
Norepinephrine (pg/ml)					
CsA	298 ± 31	-	420 ± 59	<0.05	<0.01
AZA	320 ± 37	-	364 ± 32		<0.05
Renin (μU/ml)					
CsA	40 ± 12	41 ± 12	40 ± 10	n.s.	n.s.
AZA	36 ± 5	36 ± 6	33 ± 4		n.s.
Endothelin-1 (pg/ml)					
CsA	3.8 ± 0.6	4.3 ± 0.5	5.1 ± 0.8	n.s.	<0.05
AZA	4.5 ± 0.4	4.4 ± 0.5	5.3 ± 0.5		n.s.
ANP (pg/ml)					
CsA	265 ± 53	271 ± 52	208 ± 29	n.s.	<0.05
AZA	208 ± 35	215 ± 31	183 ± 29		<0.05

Table 3. Plasma concentrations of neurohumoral factors before, during and after L-Arg infusion during CsA and AZA treatment. n.s.: not significant

DISCUSSION

In this study we found that baseline MAP was higher and GFR and RBF were lower before conversion from CsA to AZA, reflecting the well-known renal and systemic vasoconstriction associated with the use of CsA. The changes following conversion were comparable to those reported previously (21,22). Baseline plasma concentrations of norepinephrine, renin, ANP and endothelin-1 levels before and after conversion were similar, making it unlikely that these vasoactive substances play a key role in the pathogenesis of CsA-associated vasoconstriction.

Infusion of L-Arg led to a more pronounced fall in MAP and increase in heart rate during CsA than during AZA. Despite the fact that GFR and RBF were substantially lower at baseline during CsA than during AZA, L-Arg induced a more pronounced increase in GFR and RBF during CsA, resulting in comparable peak values of GFR and RBF.

Given the larger absolute and relative renal hemodynamic response to L-Arg during CsA, renal vasoconstriction during prolonged treatment with CsA can be overcome. This implies that CsA nephropathy has a considerable functional component, which apparently can be revealed by infusion of exogenous vasodilators such as L-Arg or other amino acids. A short-term increase in GFR and RBF in both animal and humans after infusion of amino acids is well-known (23,24). This renal vasodilator response has been used as a tool to determine the renal filtrating reserve capacity (25,26). Whether the application of L-Arg during CsA is a useful tool to estimate the renal reserve capacity, i.e. to predict improvement in renal function upon CsA withdrawal, remains uncertain. In our patient group the individual renal hemodynamic response to L-Arg and the improvement in graft function following conversion were not significantly correlated.

The mechanism by which L-Arg leads to vasodilatation is not entirely clear. Several studies have provided evidence that L-Arg infusion increases the production of NO through the provision of excess substrate. In other studies infusion of L-Arg at a dose comparable to that we have used, resulted in a fall in MAP and a parallel increase in exhaled NO concentration, suggesting that the systemic vasodilatation was mediated by an increase of NO production (13,27,28). Apart from its effects on NO-production, it can not be excluded that L-Arg has additional vascular effects that are not related to increased NO synthesis, such as the release of histamine or a non-specific pH effect (29).

Studies on the renal effects of infusion of L-Arg or other amino acids during CsA have yielded inconsistent results. In a study in liver transplant recipients, treatment, with

CsA did not affect the renal vasodilatation in response to infusion of a mixture of amino-acids (30). In contrast, in two cross-sectional studies performed in renal transplant recipients, who were treated with CsA or AZA, it appeared that CsA treatment which had not resulted in overt CsA nephrotoxicity, attenuated the L-Arg induced increase in renal perfusion when compared to AZA (31,32). An explanation for the discrepancy of these data and our findings is difficult to provide. In our study the patients served as their own control, giving the best possible matching between both treatments. In one other study the CsA treated patients were significantly shorter after transplantation than the AZA treated patients (31), although renal function did not differ between both groups. The fact that recently transplanted patients did not have a better renal function than the patients who were transplanted longer ago, may suggest that they were more susceptible to CsA nephrotoxicity, leading to a blunted renal vasodilation response after L-Arg infusion.

Considering *in vitro* and *in vivo* animal studies, which have shown that administration of CsA is associated with a decrease in the production of NO by endothelial cells of the renal and other vascular beds, the larger vasodilator response during CsA was unexpected. We found that the plasma level of L-citrulline, which is generated in an equimolar fashion as NO by the degradation of L-Arg, was higher during CsA than during AZA. These findings suggest that the L-Arg-NO pathway was activated rather than impaired during CsA. Activation of this pathway as a counteracting mechanism, has been reported to occur in response to endogenous vasoconstrictors such as endothelin-1, angiotensin II and norepinephrine (33,34). Since CsA has also vasoconstrictive properties, it may induce a compensatory increase in NO production. This contention is supported by a recent study reported by Stroes et al., showing an increase in NO production after administration of CsA (35).

During both treatments L-Arg had a significant diuretic effect, which ran in parallel with the increase in GFR and RBF and fall in RVR, suggesting that the renal vasodilatation contributed to the increase in urinary flow. In the late phase urine flow decreased, which probably was caused by a decrease in effective circulating volume related to the L-Arg-induced peripheral vasodilatation. L-Arg caused a twofold increase in fractional sodium excretion, which was short lived and had returned to baseline after 120 minutes. This marked increase in fractional sodium excretion may be related to the concomitant acid load caused by the infusion of L-Arg, which amounted approximately 200 mmol in this study. In healthy subjects the administration of acid enhances sodium

and net acid excretion, in order to maintain electroneutrality (36). The change in fractional sodium excretion was accompanied by a significant increase in urinary anion excretion, which we used as a parameter for urinary net acid excretion.

In conclusion, the response of systemic and renal vasodilatation after infusion of L-Arg was similar during CsA and AZA. However, the relative increase in RBF and decrease in RVR following L-Arg were substantially larger during CsA, leading to similar peak values as during AZA. This implies that chronic CsA nephrotoxicity has a substantial functional component. Our data do not support an important role for impairment of NO-mediated vasodilatation in the etiology of CsA-nephropathy and hypertension.

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SUMMARY AND CONCLUSIONS

The introduction of cyclosporine A (CsA) as the mainstay immunosuppressive drug after renal transplantation has led to much higher graft survival rates. Unfortunately, this improvement was not accompanied by a better patient survival. Possibly, the beneficial effects of CsA on graft survival are in part offset by a negative impact on risk factors for cardiovascular disease, which is the most important cause of death after renal transplantation. In view of reports from *in vitro* and animal studies suggesting this negative impact, we tried to determine whether CsA affects several newly identified risk factors for the development of cardiovascular disease. To that end, we performed a prospective study designed to analyze the impact of withdrawal of CsA as the main immunosuppressant and replacement by azathioprine (AZA). Patients who participated in this study and who were randomized for conversion from CsA to AZA, were recruited for the studies described in this thesis. In *chapter 1* various aspects of vascular medicine, that are relevant for the studies described in this thesis, are briefly reviewed. The aims of this thesis are described in *chapter 2*. In *chapter 3*, the results of the 24-hour blood pressure recordings are described. During CsA, the nighttime fall in blood pressure was significantly less than during AZA, resulting in a higher 24-hour mean arterial pressure. The diurnal variation of heart rate was not different between both treatments. Before conversion plasma ANP was higher, suggesting that volume expansion due to the sodium retaining effects of CsA may play a causative role in the disturbance of the diurnal pressure rhythm.

In *chapter 4*, healthy controls and CsA-treated renal transplant recipients are compared with regard to lipoprotein profile and *in vitro* and *in vivo* LDL composition and oxidation. Renal transplant patients appear to have higher plasma levels of total cholesterol and triglycerides. The LDL particles of these patients are smaller and more susceptible to oxidation, both *in vitro* and *in vivo*.

As described in *chapter 5*, the plasma level of both total cholesterol and triglycerides decreased significantly after withdrawal of CsA and conversion to AZA. In addition, the LDL particle size increased markedly, which was also illustrated by a reduction in the number of patients with the atherogenic LDL subclass B. LDL was significantly less susceptible to oxidation after conversion. These results suggest that CsA facilitates the oxidation of LDL, possibly through elevation of plasma triglyceride levels.

The impact of conversion on fibrinolytic activity is described in *chapter 6*. During CsA plasma levels of PAI-1 activity and antigen concentration were higher than during AZA,

resulting in impaired tPA activity, while tPA antigen concentration was unchanged. Plasma levels of the prostanoids PGE_2 and TxB_2 were substantially elevated during CsA. Interestingly, during CsA but not AZA, these plasma levels were significantly correlated to PAI-1 and tPA concentration and activity. This may suggest that the increased incidence of vascular events which has been linked to the use of CsA, is caused by a prostanoid-mediated impairment of fibrinolytic activity.

The integrity of endothelial vasodilator function was investigated in the forearm model and in the renal allograft. In *chapter 7*, the results of the forearm studies are given. The response to the endothelium-dependent vasodilator acetylcholine and to the endothelium-independent vasodilator sodiumnitroprusside, were not different between CsA and AZA, despite the fact that arterial pressure was higher during CsA.

The systemic and renal effects of intravenous administration of L-arginine are described in *chapter 8*. During CsA the blood pressure lowering effect of L-arginine was more pronounced than during AZA. Although baseline values were lower, L-arginine caused a larger absolute and relative increase in glomerular filtration rate and renal blood flow during CsA, indicating that even after prolonged use of CsA, renal vasoconstriction is largely functional. The renal response after L-arginine infusion did not predict the improvement in graft function after CsA withdrawal. No changes occurred in plasma levels of vasoactive compounds such as renin, endothelin-1 or norepinephrine, both following conversion or L-arginine infusion. These data suggest that in renal transplant recipients with CsA-induced hypertension and renal vasoconstriction, endothelial vasodilator function is preserved (forearm) or even enhanced (renal allograft). Therefore, other mechanisms must be responsible for the development of these adverse events.

We conclude that the use of CsA is accompanied by impaired 24-hour blood pressure variation, increased susceptibility of the oxidation of LDL and also impairment of fibrinolyitc activity, which are all recently identified risk factors for the development of cardiovascular disease. Conversion from CsA to AZA, is a method to ameliorate the cardiovascular risk profile of renal transplant patients and may improve the long-term prognosis of renal transplant recipients.

SAMENVATTING EN CONCLUSIES

De introductie van cyclosporine A (CsA) in het begin van de tachtiger jaren heeft de resultaten van niertransplantatie zeer duidelijk verbeterd, waardoor dit geneesmiddel nu een centrale plaats inneemt bij het voorkomen van transplantaatafstoting. Deze gunstige ontwikkeling in transplantaatoverleving is echter niet gevolgd door een verbetering van de patiëntenoverleving. Dit zou verklaard kunnen worden doordat de gunstige invloed van CsA op de transplantaatoverleving deels teniet wordt gedaan door een negatieve invloed van CsA op risicofactoren voor hart-en vaatziekten, die immers de belangrijkste doodsoorzaak zijn na niertransplantatie. Een dergelijke negatieve invloed is al eerder gevonden bij laboratorium- en proefdieronderzoek. Daarom richtte het beschreven onderzoek zich op het bestuderen van de invloed van het gebruik van CsA op enkele recent ontdekte risicofactorren voor hart-en vaatziekten bij niertransplantatiepatiënten.
Derhalve werd een prospectief onderzoek opgezet, gericht op het analyseren van de effecten van het vervangen van CsA door een ander afweeronderdrukkend middel, azathioprine (AZA).
In *hoofdstuk* 1 zijn is de achtergrond van de verschillende cardiovasculaire risicofactoren samengevat. In *hoofdstuk 2* zijn de doelen van de verschillende studies kort weergegeven. De resultaten van de semicontinue 24-uurs bloeddrukmetingen zijn beschreven in *hoofdstuk 3*. Tijdens behandeling met CsA bleek de over 24 uur gemiddelde bloeddruk duidelijk hoger dan tijdens AZA. Daarnaast was de bloeddrukdaling die normaal gedurende de nacht optreedt, tijdens CsA behandeling belangrijk verminderd. De bevinding dat de gemiddelde plasmaconcentatie van atriaal natriuretisch peptide, een hormoon wat gebruikt wordt als maat voor de hoeveelheid vocht in het lichaam, hoger was tijdens CsA, suggereert dat verminderde zoutuitscheiding hierbij een rol speelt. De dag-nacht variatie van de hartfrequentie veranderde niet onder invloed van CsA behandeling.
In hoofdstuk 4 werd de verschillen onderzocht tussen met CsA behandelde niertransplantatiepatiënten en gezonde proefpersonen, met betrekking tot hun plasma lipidenprofiel, de samenstelling en mate van oxidatie van lipiden in plasma, en de gevoeligheid voor oxidatie. Bij niertransplantatiepatiënten bleken de concentraties van cholesterol en triglycerides aanzienlijk hoger dan bij gezonden. Tevens was het LDL-cholesterol veel gevoeliger voor oxidatie, wat het ontstaan van atherosclerose vergemakkelijkt. Zoals beschreven in *hoofdstuk 5*, resulteert het vervangen van CsA door AZA in een aanmerkelijke daling van het plasma cholesterol en triglyceriden gehalte.

Daarnaast waren tijdens CsA behandeling LDL deeltjes kleiner, en daarom gevoeliger voor oxidatie, dan tijdens AZA behandeling. Dit wijst erop dat CsA een negatieve invloed heeft op het ontstaan van atherosclerose door bevordering van lipidenoxidatie, wat mogelijk wordt veroozaakt door de ongunstige invloed van CsA op het triglyceriden gehalte in plasma.

De invloed van behandeling met CsA op het fibrinolytisch systeem, cruciaal bij het voorkomen van bloedstolling, is beschreven in *hoofdstuk 6*. Gedurende behandeling met CsA waren de plasma concentraties van PAI-1, de belangrijkste regulator van het fibrinolytisch systeem, duidelijk hoger dan gedurende AZA behandeling. Dit ging gepaard met een verminderde activiteit van de plasminogen activator tPA, terwijl de plasma concentratie niet verschilde tussen CsA en AZA. De plasmaconcentraties van de prostanoiden TxB_2 en PGE_2 waren hoger tijdens CsA dan tijdens AZA. Deze concentraties waren alleen tijdens behandeling met CsA, maar niet met AZA, significant gecorreleerd met de concentratie en activiteit van PAI-1 en tPA. Dit wijst erop dat verstoring van het prostanoidenmetabolisme ten grondslag ligt aan de verminderde fibrinolyse tijdens CsA behandeling.

Endotheelcellen spelen een cruciale rol bij de interactie tussen bloed en vaatwand, zijn mede bepalend voor de vaatwandtonus. De functie van deze cellaag, die ook uitermate belangrijk is bij het ontstaan van atherosclerose, werd op twee manieren onderzocht. In het onderarmsmodel werd de reactie van het vaatbed in de onderarm op intra-arteriele toediening van zowel endotheel-afhankelijke, als endotheel-onafhankelijke vaatverwijdende middelen bestudeerd. De responses op beide soorten vaatverwijders was niet verschillend tussen CsA en AZA behandeling, wat erop wijst dat vermindering van endotheel functie geen belangrijke rol speelt bij het ontstaan van hoge bloeddruk tijdens CsA behandeling. De resultaten van dit onderzoek zijn weergegeven in *hoofdstuk 7*. Om de vaatverwijdende capaciteit van het niervaatbed te bepalen werd het aminozuur L-arginine intraveneus toegediend, waarna de veranderingen in bloeddruk, hartfrequentie, maar ook nierdoorstroming en urineproduktie werden gemeten, zoals beschreven in *hoofdstuk 8*. Wij vonden dat tijdens CsA de daling van de bloeddruk meer uitgesproken was dan tijdens AZA. Opvallend was dat ondanks de lagere uitgangswaarden van glomerulaire filtratiesnelheid en nierdoorstroming, L-arginine toediening tijdens CsA gevolgd werd door een grotere absolute en relatieve toename van deze parameters. Dit wijst erop dat de nierfunctievermindering tijdens CsA voor een belangrijk deel functioneel,

en reversibel is. Een verband tussen de toename van de hemodynamische parameters, en de verbetering na het vervangen van CsA door AZA, kon niet worden aangetoond.
De plasma concentraties van vasoactieve hormonen als renine, noradrenaline, en endotheline-1, werden niet beinvloed door het vervangen van CsA door AZA, als door infusie van L-arginine. Ook in de nier lijkt dus verminderde endotheel-gemediëerde vaatverwijding geen belangrijke rol te spelen bij het ontstaan van nierunctie vermindering door CsA.
Concluderend leidt het gebruik van CsA tot een verstoring van de 24-uurs bloeddrukvariabiliteit, een toename van de oxidatie van LDL-cholesterol, en een vermindering van de fibrinolytische activiteit. Het ongunstige effect van CsA op deze sinds enkele jaren onderkende risicofactoren, kan bijdragen tot het frequent voorkomen van hart-en vaatziekten bij niertransplantatiepatiënten. Vervangen van CsA door AZA is een mogelijkheid om het cardiovasculair risicoprofiel, en daarmee de prognose, bij deze patiëntengroep te verbeteren.

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CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 24 april 1963 te Tholen. In 1981 behaalde hij het VWO-B diploma aan de Rijksscholengemeenschap te Bergen op Zoom. Aansluitend startte hij met de studie Geneeskunde aan de Erasmus Universiteit te Rotterdam. In periode 1984 tot 1987 was hij student-assistent op de Afdeling Celbiologie van de EUR. In 1988 behaalde hij het artsendiploma (cum laude). Van 1988 tot 1994 werkte hij als arts-assistent in opleiding tot internist op de Afdeling Interne Geneeskunde I van het Academisch Ziekenhuis Rotterdam- Dijkzigt (opleider: Prof. dr M.A.D.H. Schalekamp). Na de registratie als internist per 1 oktober 1994, volgde hij het aandachtsgebied Nefrologie in het AZR (opleider: Prof. dr W. Weimar). Per 1 januari 1997 werd hij als internist-nefroloog registreerd, en vanaf 1 juni 1997 is hij als zodanig werkzaam in het St.Clara Ziekenhuis te Rotterdam.