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ORIGINAL ARTICLE

Pharmacodynamic cyclosporine A-monitoring: relation of gene expression in lymphocytes to cyclosporine blood levels in cardiac allograft recipients

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Summary

Recently, we established a pharmacodynamic assay to monitor immunosuppressive effectiveness of cyclosporine A (CsA) in patients on standard CsA regimen. The aim of the present study was to extend this correlation to reduced CsA regimen and to compare pharmacodynamic and kinetic parameters to allow prediction of rejections and infections. In 53 heart allograft recipients, nuclear factor of activated T cells (NFAT)-regulated gene expression was quantified at trough (C0) and 2-h post-CsA dose (C2). Gene expression at C2 was calculated relative to C0 (residual gene expression, RGE) or relative to a healthy reference group (absolute gene expression, AGE). RGE correlated with CsA C2-levels in bimodal fashion: above 575 ng/ml correlation was seen with flat regression gradient. Below 575 ng/ml, correlation was excellent with markedly steeper gradient. At C0 in the low-C2 group (<575 ng/ml), AGE remained unchanged, whereas in the high-C2 group (>575 ng/ml) AGE was markedly reduced. In both groups, AGE at C2 was strongly inhibited. In patients contracting infection during follow-up, RGE was lower than in those without infections independent of CsA levels. CsA-monitoring by quantitation of NFAT-regulated gene expression is feasible with standard and reduced CsA regimens. It correlates better with the incidence of infections than measurement of CsA concentrations and might help in avoiding over-immunosuppression.

Introduction

CyclosporineA (CsA) is one of the pivotal components in various immunosuppressive regimens after organ transplantation. To minimize the potential complications of immunosuppression in transplantation medicine, e.g. infections and rejections, drug monitoring is essential [1,2]. Currently, mainly pharmacokinetic assays [drug concentrations, area under the concentration–time curve (AUC)] are used [3–8]. Also, novel software has been developed to optimize individual AUC estimation [9]. To allow pharmacodynamic assessment of drug action, T-cell activation processes under medication would need to be studied in a quantitative fashion potentially allowing more sensitive assessment of individual immunosuppression. To date, some approaches to monitor CsA pharmacodynamically have been made on protein (phosphatase activity and cytokine synthesis), cellular (proliferation) as well as mRNA level [10–18].

We reported recently that measuring expression of nuclear factor of activated T cells (NFAT)-regulated genes in phorbol-12-myristate-13-acetate (PMA)/ionomycin-stimulated peripheral blood cells by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) can be used to monitor biologic activity of CsA *ex vivo* [19]. A direct correlation between cyclo-

sporine concentration and inhibition of gene transcription for interleukin-2 (IL-2), granulocyte-monocyte colony stimulating factor (GM-CSF) and interferon-γ (IFN-γ) was seen. Maximum inhibition was demonstrated for the highest drug levels 2 h after intake (C2 levels). In the actual dose range of these patients, inhibition of transcription was almost complete in all but a few patients, resulting in a flat dose–effect relation.

The aim of the present study was to extend the correlation between C2 level and inhibition of gene expression to patients on reduced-dose CsA regimen. Furthermore, we tested whether patients suffering infections or rejections differ in their pharmacokinetic (C0, C2) and pharmacodynamic parameters.

Materials and methods

Characteristics of the heart transplant patients

Patients ($n = 53$) were included between June 2002 and August 2003 at the outpatient clinic at University of Heidelberg. At the time of enrolment, gene expression assay was performed and pharmacokinetic parameters were assessed. Only those patients who were free from acute infection and who showed stable organ function without rejection at the time of enrolment were included. Patients had to be on stable CsA therapy for at least 4 months. CsA reduction was predominantly instituted for the prevention of further deterioration of renal function (nephroprotection). Target trough blood level in these patients was between 50 and 100 ng/ml. Participating subjects gave informed consent under a protocol approved by the institutional review board in accordance with the Declaration of Helsinki. The mean follow-up was 16.7 ± 2.3 months. A total of 13.2% were female, mean time after transplantation was 90.3 ± 7.3 months. Twelve patients were on a triple, CsA-based regimen, [11 with mycophenolate mofetile (MMF) and steroids, one with AZA (azathioprine) and steroid]. Twenty-four patients received MMF, 11 AZA and six steroids additionally to CsA as dual immunosuppressive regimen (Table 1). Study subjects were on stable immunosuppression during follow-up, no changes in dose of cyclosporine or additional immunosuppression of >15% were instituted during the observation period.

After selection of the patients at baseline, clinical follow-up was analysed retrospectively by database investigation. Gene expression analysis was performed at baseline; for CsA monitoring during follow-up, only pharmacokinetic parameters were available. Follow-up consultations were performed regularly and biopsies were taken according a standardized protocol [20]: weekly in the first month; twice during month 2; every 4 weeks during months 3–6; every 8 weeks during months 6–12. In post-transplant years 2 and 3, routine biopsies were performed twice annually, and thereafter biopsies were performed once yearly or upon clinical suspicion of rejection. For the first year, outpatient visits are scheduled following the biopsy-protocol, afterwards visits are on a 4-month basis or in case of any clinical irregularity. Experienced cardiac pathologists graded the biopsy specimens according to International Society for Heart and Lung Transplantation (ISHLT) criteria uninformed of this study. Blood samples were drawn predose (C0) and 2 h (C2) after oral administration of CsA. Rejection incidence was calculated for each patient as number of rejections per month of the observation period. The incidences were calculated following the old as well as the new ISHLT grading system separately for ISHLT ≥ 2, ISHLT ≥ 3A, ISHLT ≥ R1, and ISHLT ≥ R2. Rejection nomenclature of 1990 ('old' version) [21] is predominantly used as patient

Table 1. Clinical/demographic characteristics of 53 study patients, separated by high vs. low cyclosporine C2 level. The mean of each character is shown and has been tested for significant difference using the Mann–Whitney test. The incidence of rejections has been calculated following the new as well as the old scoring system of the International Society for Heart and Lung Transplantation (ISHLT). Incidence is shown as number rejections per month of the observation period. The both groups are comparable but the cyclosporine A (CsA) dosage and blood levels at C0 and C2.

	High C2 ($n = 24$)	Low C2 ($n = 29$)	P
Mean age, years (range)	61.6 (46–70)	59.3 (30–76)	n.s.
Sex: M/F	20/4	26/3	n.s.
Days post-HTX	2282	3060	n.s.
CsA (mg/kg/day) ± SD	2.72 ± 0.15	2.1 ± 0.15	$P < 0.05$
C0 (ng/ml) ± SD	141 ± 13	94.4 ± 8	$P < 0.01$
C2 (ng/ml) ± SD	817.5 ± 41.9	404 ± 24.7	$P < 0.0001$
Mycophenolate mofetile (number, mg/kg/day ± SD)	16, 36.7 ± 2.9	19, 35.6 ± 1.8	n.s.
Azathioprine (number, mg/kg/day ± SD)	7, 0.88 ± 0.13	6, 0.69 ± 0.24	n.s.
Rejections ISHLT ≥ 2 (incidence ± SD)	0.03 ± 0.01	0.02 ± 0.007	n.s.
Rejections ISHLT ≥ 3A (incidence ± SD)	0.01 ± 0.006	0.004 ± 0.002	n.s.
Rejections ISHLT ≥ R1 (incidence ± SD)	0.08 ± 0.02	0.06 ± 0.02	n.s.
Rejections ISHLT ≥ R2 (incidence ± SD)	0.01 ± 0.006	0.004 ± 0.002	n.s.

enrolment and follow-up/analysis occurred exclusively before the introduction of the revised classification of 2005 [22].

Infection was diagnosed by clinical symptoms, c-reactive protein (CRP) levels and microbial culture of body fluids. Clinical symptoms or findings were diagnostic for an infectious event in 18.9% of cases (five patients: bronchopulmonary infection, three patients: gastroenteritis, one patient: cytomegalie viremia, one patient: positive urine culture). Pathological CRP levels were considered infection-related, if they rose acutely to above four times the normal value, reasons for elevated CRP other than infection could be excluded (trauma, operation, tumor, rejection, etc.) and routinely measured CRP levels immediately before the infectious episode were normal and resolved in the subsequent follow-up. In eight out of the 10 cases, patients were treated with antibiotics, the remaining two infections were due to a severe viral respiratory illness.

Cyclosporine A concentrations were determined by commercial TDM assay (DF89, Dade-Behring, Marburg, Germany) [23]. Adequate mycophenolate dosage was ascertained determining predose levels (aimed at >1.0 mg/l). Plasma concentrations were measured with a validated EMIT Mycophenolic Acid Assay (Dade-Behring) [24]. In borderline cases, monitoring was complemented by 3-point AUC estimations aimed at >40 mg/l × h [25]. Azathioprine was given at 2–4 mg/kg bodyweight under control of leukocyte count >3500/μl.

Sample preparation

Heparinized venous peripheral whole blood (1 ml) was stimulated with 1-ml RPMI-1640 Medium (Gibco, Karlsruhe, Germany) containing 100 ng/ml PMA (Sigma, Taufkirchen, Germany) and 5 μg/ml ionomycin (Sigma) for 3 h at 37 °C. mRNA was isolated with MagNAPure-LC device using mRNA standard protocol for cells and reverse-transcribed using first-strand cDNA synthesis kit (Roche, Mannheim, Germany) in a thermocycler.

Quantitative analysis of gene expression

Nuclear factor of activated T cells-regulated gene expression (IFN-γ, IL-2, and GM-CSF) was quantified using qRT-PCR with the LightCycler [19]. Briefly, target sequences were amplified using commercially available LightCycler Primer-sets (Search-LC, Heidelberg) with the LightCycler FastStart DNA Sybr-Green-I Kit (Roche Diagnostics). Transcript concentration for the measured genes were calculated from a external standard curve, obtained by blotting a known input concentration of a plasmid to the PCR cycle number at witch the detected fluorescence intensity reaches a fixed value. To correct for variations in the yield, samples were normalized to the expression of two housekeeping genes (β-actin and cyclophilin B). The data of the two independent analyses for each sample and parameter were averaged and presented as adjusted transcripts/μl cDNA. NFAT-regulated genes were analyzed before (C0) and 2 h after (C2) CsA intake. For each cytokine (IL-2, GM-CSF and IFN-γ), gene expression at C2 was calculated relative to the corresponding gene expression at C0. Mean of these three relative values of gene expression at C2 was considered the patients' 'residual gene expression' (RGE). For the normalized absolute gene expression (AGE), the gene expression of each cytokine was calculated relative to the mean gene expression of the respective cytokine in a healthy reference group. For each patient, the mean of these three normalized values was considered the patients' AGE. AGE can be calculated at C0 as well as C2 relative to this reference group. This reference group consisted of 34 healthy individuals between 25 and 45 years of age, 50.9% female. They were free from infection and without any immunosuppressive medication. The coefficient of variation between the same sample run repeatedly of this method is below 11%. Intra-individual variability is 9%; repeated determination in the same patient at a second time point correlates strongly ($r = 0.77$, $P < 0.001$; $n = 82$). Inter-individual variability of RGE is 14% ($n = 31$, all patients with 100 mg CsA/day) [26].

Statistics

Data were analyzed by the Mann–Whitney, Wilcoxon, and chi-squared test using SPSS 11.0 (Mac OsX; SPSS, Chicago, IL, USA) statistical software package. For computer assisted curve fitting analysis, SPSS 13.0 was used.

Results

NFAT-regulated gene expression is inhibited at peak CsA concentration (C2)

Residual gene expression was calculated [19] in 53 heart allograft recipients. Figure 1 shows the inverse relation of mean RGE 2 h after drug intake (C2) compared with mean CsA levels in these patients: mean trough CsA level was 115.5 ± 8.15 ng/ml, increasing to 591.36 ± 36.7 ng/ml after 2 h, whereas mean RGE decreased to 26.52 ± 3.2% relative to C0 (set as 100% as defined, $P < 0.0001$).

Correlation between gene expression and CsA levels

In 29 of 53 patients, CsA dosage had been reduced due to nephrotoxicity resulting in a wide overall range of C2 levels between 140 and 1255 ng/ml. RGE was correlated with

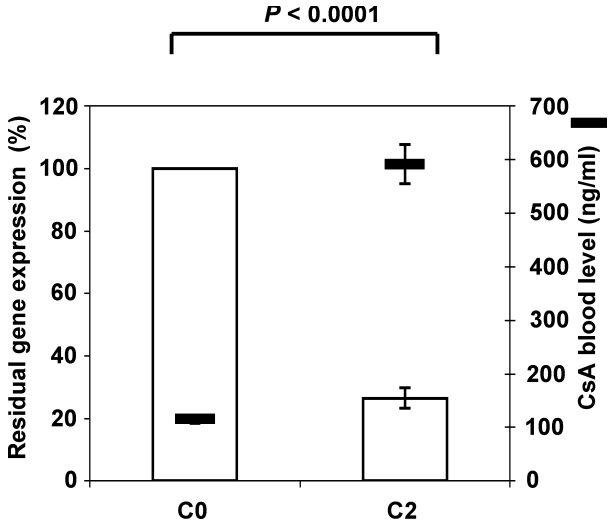

Figure 1 Residual gene expression (RGE) and cyclosporine A (CsA) levels before and 2 h after drug intake. Peripheral blood cells of 53 cardiac allograft recipients were stimulated with phorbol-12-myristate-13-acetate /ionomycin before (C0) and 2 h after CsA intake (C2). Selected nuclear factor of activated T cells-regulated gene expression (IL-2, GM-CSF and IFN-γ) was analyzed using quantitative reverse transcriptase polymerase chain reaction. The right *y*-axis shows the mean CsA blood level (line, ng/ml), the left *y*-axis (%) shows the RGE. Bars show the SE of the mean. At C2 RGE is significantly reduced to 26.52 ± 3.2% relative to C0 ($P < 0.0001$), whereas the mean CsA level increases from 115.5 ± 8.15 ng/ml at C0 to 591.36 ± 36.7 ng/ml after 2 h.

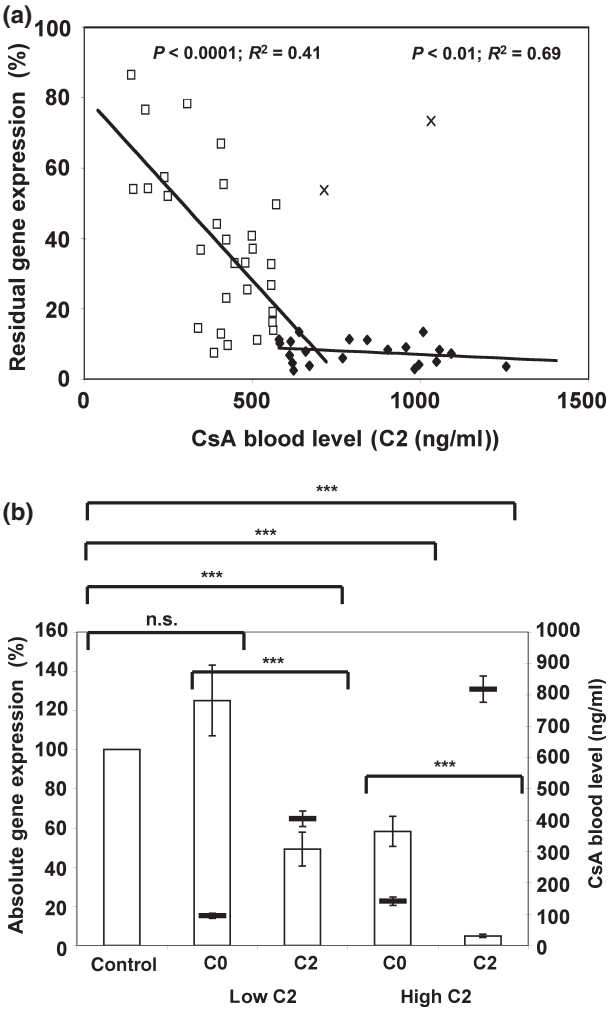

Figure 2 Correlation between gene expression and cyclosporine A blood levels. (a) The graph shows the distribution of 53 samples of cardiac allograft recipients ranging between 115 und 1255 ng/ml for C2 levels, *y*-axis indicates corresponding residual gene expression (RGE). Regression analysis clearly shows that RGE is directly related to C2 levels in a biphasic fashion. R^2 and *P*-values are calculated for all 53 patients except the two outliers marked by a cross. (b) The graph shows the mean absolute gene expression (AGE) at C0 and C2 separately for 'high and low C2' (left *y*-axis). Also, the corresponding mean CsA blood levels (line, ng/ml; right *y*-axis) for both groups and time-points are depicted. Bars show the SE of the mean. For AGE control compared with C0 in the low C2 group was not significantly different, whereas all other samples (C2 in the low C2 group, C0 and C2 of the high C2 group) were significantly reduced compared to control ($P < 0.0001$). Also, within each group, low and high C2- AGE at C2 is significantly reduced compared with the corresponding AGE at C0 ($P < 0.0001$). ***$P < 0.0001$.

C2 CsA levels (Fig. 2a). After computer-assisted curve fitting, data-points were described best by a sigmoid graph, which can be approximated by two linear regressions, dividing patients into two groups with C2 levels above and below 575 ng/ml, considered 'high' and 'low' CsA. Above 575 ng/ml, RGE ranged from 13.4% to 2.5% and correlated with C2 levels by way of a flat regression gradient ($P < 0.01$; $R^2 = 0.069$). Below 575 ng/ml, RGE varied between 86.4% and 7.4% and was also closely related with C2 levels ($P < 0.0001$; $R^2 = 0.41$), however with a considerably steeper gradient (Fig. 2a). In a small number of patients, CsA concentration and RGE were discrepant to the calculated regression graphs [e.g. RGE of 73.3% and 53.7% despite high C2 levels of 714 and 1032 ng/ml (crosses in Fig. 2a)].

To evaluate further, the status of immunosuppression normalized AGE was calculated (Fig 2b). AGE for all patients was marginally inhibited at C0 (94.8 ± 11.36%; $P < 0.05$) and strongly inhibited at C2 (29.1 ± 5.61%; $P < 0.0001$) (not shown). When analysing AGE separately for patients with high versus low C2 concentrations, the latter had very low C0 concentrations (94.4 ± 8.2 ng/ml) and displayed un-inhibited gene expression (125.1 ± 18.1%; n.s.) at trough compared with nontreated reference group. At C2 in this group, mean CsA level increased to 404.1 ± 24.7 ng/ml and AGE at C2 decreased to 49.1 ± 8.6% ($P < 0.0001$). In contrast, in patients with C2 concentrations above 575 ng/ml, AGE was already

significantly lower at C0 (58.2 ± 7.7%; $P < 0.0001$) and decreased further at C2 (4.9 ± 0.8%; $P < 0.0001$). In this group, mean CsA levels were 141 ± 13.4 ng/ml at C0 and 817.5 ± 41.9 ng/ml at C2.

Absolute gene expression at C2 also correlated inversely with CsA C2 levels in a bimodal fashion. Above 575 ng/ml, AGE values ranged between 17.4% and 0.61% at C2 with a quite flat regression curve ($P < 0.05$; $R^2 = 0.039$). Below 575 ng/ml (low C2), AGE levels ranged between 194.77% and 4.26% and correlated in a steeper regression curve ($P < 0.0001$; $R^2 = 0.24$). In those outliers showing high RGE despite adequate C2 concentration, AGE was already highly suppressed at C0 (7.5% at 192 ng/ml C0 CsA and 6.8% at 125 ng/ml C0 CsA). Consequently, by this data treatment (AGE), the outlier data points disappear (not shown).

Patients with infectious complications show reduced RGE

To compare pharmacokinetics and gene expression as predictors of adverse events of immunosuppression (rejection and infection), CsA levels and RGE were related to clinical events during follow-up. Patients were subdivided into groups of rejectors versus nonrejectors and in subjects with acute infections versus without infections over a 2.25-year observation period. RGE and CsA levels, respectively, were compared for their ability to discriminate between groups.

In the observed period, 38 rejections ISHLT ≥ 2 in 21 different patients and 11 rejections ISHLT ≥ 3A in eight individual patients were documented. Neither C2 levels nor RGE correlated with the incidence of rejections (separate analyses for ISHLT ≥ 2 or ISHLT ≥ 3A) in our collective.

Importantly, RGE was significantly lower in patients contracting an infection during the follow-up period (13% vs. 28.7%; $P < 0.05$) whereas C2 concentrations were not significantly different between patients with or without infection (Fig. 3a and b). Similarly, no correlation was found for C0 CsA concentrations with either rejection or infection.

Importantly, there was no significant difference in additional immunosuppressive medication when comparing patients with and without infectious complications or in the comparison of patients with and without rejections of grade ISHLT ≥ 3A (=2R of new nomenclature). Patients with rejections ISHLT ≥ 2 (old nomenclature) showed significantly increased dosage of MMF (32.75 ± 3.8 mg/kg/day vs. 19.7 ± 3.3 mg/kg/day; $P < 0.05$) and steroids (0.04 ± 0.01 mg/kg/day vs. 0.008 ± 0.004 mg/kg/day) compared with nonrejectors (data not shown).

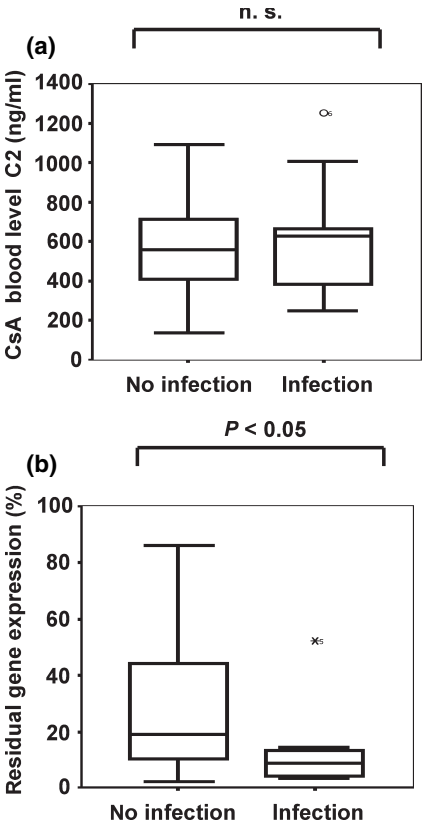

Figure 3 Residual gene expression (RGE) is significantly reduced in patients contracting infections during follow-up. Patients were divided into two groups according the incidence of infection, subsequently the mean of C2 levels (a) and of RGE were calculated (b). Then for each parameter, the mean of both groups was tested for a significant difference. Box plots and median are depicted.

Discussion

In this study, we show that pharmacodynamic monitoring of CsA effectiveness, using a recently published qRT-PCR-based assay [19], is feasible over a large range of CsA concentrations and that it appears to correlate better with the risk of infections than standard determination of CsA levels (C0, C2).

Excellent bimodal correlation between CsA C2-level and gene expression data could be found. The regression gradient in the high C2 group was found to be flat, suggesting that reduction of immunosuppressive dosage may be possible while maintaining similar biological effect. Supporting this thesis, the incidence of rejection was not elevated in the low C2 group, suggesting that complete suppression of T-cell activation may not be required and adverse effects could be minimized by further reduction of immunosuppression. Besides a reduced infection risk, improvement of renal dysfunction could be achieved by

further reduction of CsA [27]. It is important to note that all patients received other immunosuppressive drugs additionally to CsA. However, there was no significant difference in the dose of this additional medication between both groups. Low incidence of rejection episodes in patients on reduced CsA dose (low C2) might be in part due to a selection bias as low-dose CsA regimens tend to be implemented more readily in patients with benign rejection profile; however, no differences were found in the incidence of rejection between the groups for the early phase post-op. or for the entire period after transplantation rendering such bias unlikely.

Currently, several approaches to monitor CsA pharmacodynamically have been made [10–18]. However, none of these assays has been implemented in routine clinical practice. The test herein presented provides a reliable and robust quantification of functional immunosuppression at the target cell level. It is almost completely automated in a specialized laboratory and provides results within 1 day at relatively low costs. This assay is whole blood based and needs no time-consuming cell separation. PMA/ionomycin-induced RGE depends only on CsA dose and is independent of comedication, allowing isolated monitoring of CsA effectiveness [19]. Additional immunosuppression reduces the basal NFAT-regulated gene expression at C0 when compared with a healthy reference group, but the reduction of gene expression between C0 and C2 (i.e. RGE) is entirely CsA-specific. This can also be seen, when C2 levels in the correlation of Fig. 2a are extrapolated towards 0 ng/ml: RGE approximates to 100%, which means no inhibition of RGE without CsA.

Gene expression data can be presented in different ways: as RGE at C2 relative to the individual gene expression at C0 or as AGE in relation (normalized) to a non-immunosuppressed reference standard. AGE determination may help separate patients that are insufficiently immunosuppressed as a consequence of nonresponse to CsA from those that are already significantly inhibited at C0 (false nonresponders). However, AGE shows broad interindividual variation and is in contrast to RGE not CsA-specific.

To test the clinical relevance of C2 level monitoring versus gene expression, the incidence of infections and rejections were analyzed. For rejection episodes, no correlation could be found with either monitoring modality. This lack of association may partly be explained by the low rejection incidence to be expected in the observation period at the chronic maintenance stage (>2 years post-transplantation in most patients) [28]. In fact, the incidence of rejections in the observation period was not low (21 patients with rejection ISHLT ≥ 2 and eight with ISHLT ≥ 3A). However, these findings need to be extended to patients in the early post-transplant phase. In the study cohort for patients with rejections ISHLT ≥ 2 (old nomenclature), increased dosage of additional immunosuppressive medication (MMF and steroids) could be demonstrated, which at least partly may explain the lack of a statistical association. In contrast, for infection risk during the follow-up phase, a significant difference between gene expression analysis and standard CsA concentration measurement could be found in this small pilot-study: RGE appeared to be a significantly better predictor of infections than C2 level monitoring. Importantly, in the comparison of patients with and without infections, no significant difference in additional immunosuppressive medication could be found. Controlled prospective studies including higher numbers of patients are needed to confirm the advantage of individualized pharmacodynamic over pharmacokinetic drug monitoring regarding clinical outcome; in patients with very low RGE, CsA dosage might be reduced provided that graft situation is stable and the patients history is free of multiple rejections. Interestingly, a similar association was found in renal allograft recipients, supporting the relevance/robustness of the herein presented assay in principle [26].

While generally no direct association of cyclosporine C0 levels could be shown with either rejection or infection, several recent reports describe better correlations of C2 concentrations with clinical events, primarily rejection, in renal and heart transplant recipients [4,29,30]. These data are, however, inconsistent in that some patient cohorts display higher C2 levels in rejecting patients [31], whereas others show higher C2 concentrations in nonrejectors for adult as well as pediatric heart transplant recipients [32–34]. A reliable association with infectious complications has thus far not been reported with C2 level monitoring; also in the present study no correlation of C2 levels with infections was seen.

In conclusion, this work represents a pilot study towards pharmacodynamic CsA monitoring at the target cell level. Here, we have shown that a gene expression assay can be applied over a wide range of C2 levels. It shows excellent, bimodal correlation between C2 levels and gene expression data (AGE and RGE). Using this assay, an individual patient's likely target C2 level can be determined. For daily routine, C2 level measurement appears sufficient because of this good correlation. Whereas RGE represents a CsA-specific marker, AGE allows evaluation of the immunosuppressive status relative to a healthy reference group. Interestingly, in this small pilot study the RGE correlates better with infectious complications than pharmacokinetic parameters (C0, C2).

Authorship

MK, TD wrote the paper; MK, CS, TG analyzed data; AD collected data; MZ, SM, HK, TD, TG designed research study.

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