Transplant International

Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

Correlation of tacrolimus levels in peripheral blood mononuclear cells with histological staging of rejection after liver transplantation: preliminary results of a prospective study

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Keywords
intrahepatic concentration, liver transplantation, peripheral blood mononuclear cells, rejection, tacrolimus.

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Conflicts of Interest
The authors have declared no conflict of interest.

Received: 18 April 2011
Revision requested: 4 May 2011
Accepted: 12 September 2011
Published online: 8 October 2011

doi:10.1111/j.1432-2277.2011.01365.x

Summary

Therapeutic drug monitoring of tacrolimus (TAC) is characterized by a complex relationship between trough blood TAC concentrations and therapeutic efficacy. This prospective study evaluates the predictive value of intrahepatic, peripheral blood mononuclear cells (PBMCs) and blood TAC concentrations during the early postliver transplantation (LT) period. In a cohort of 90 adult liver recipients under TAC-based monotherapy, liver biopsies were performed at day 7 post-LT, and PBMCs TAC concentrations were measured at day 1, 3, 5, and 7 post-LT. Both intrahepatic and PBMCs TAC concentrations were determined. All biopsies were graded following the Banff scoring. Intrahepatic, and day 3, 5, 7 PBMCs concentrations correlated very well with day 7 liver Banff rejection scores ($P < 0.05$). Clinical rejection was characterized by significantly lower mean TAC PBMCs concentrations at day 5 and 7 ($P < 0.05$) and tended to be associated to lower mean intrahepatic TAC concentrations at day 7 ($P = 0.059$). Intrahepatic TAC concentrations at day 7 significantly correlated with TAC PBMCs concentrations from day 5 post-LT ($P < 0.05$). TAC PBMCs concentrations might be reliable markers of immunosuppression efficacy during the early phase after LT. This finding could represent an additional tool to individualize more precisely early immunosuppressive schemes after liver transplantation.

Introduction

Tacrolimus (TAC) is nowadays considered as the cornerstone immunosuppressant in liver and kidney transplantation [1]. As a result of the narrow therapeutic ranges and to large pharmacokinetic, -dynamic, and -genetic inter-patient variability, therapeutic drug monitoring (TDM) remains mandatory to optimize clinical outcome and reduce toxicity [2]. However, the relationship between TAC blood concentrations and the incidence of graft rejection is unclear, as published results are contradictory [3–7]. Better strategies for drug optimization in allograft recipients are therefore needed; these could include identification and validation of pharmacodynamic biomarkers and direct drug measurement at the target sites, i.e., allograft tissue [8–10] and lymphocytes [11–13].

Our previous experimental work in liver transplantation (LT) showed that low TAC allograft tissue exposure, in contrast to the trough blood concentrations, was associated with significantly higher incidence of graft rejection

[8]. The immunosuppressive effect of TAC is mediated through the inhibition of calcineurin in lymphocytes; a closer link to drug efficacy could therefore be expected from direct quantification within this target compartment, as compared with whole blood or even tissue concentrations.

The present study investigated the relationship between pre-dose TAC concentrations in peripheral blood mononuclear cells (PBMCs), representing a blood compartment enriched with lymphocytes, and the severity of the cellular rejection after LT under a well defined and standardized study protocol.

Material and methods

Study population

During the period from November 2008 to August 2010, 90 adult (>15 years) patients underwent isolated, primary LT for chronic end-stage liver disease at the Cliniques universitaires St-Luc in Brussels. There were 63 men versus 27 women with a median age of 52.5 years: (range from 20 to 68). These patients were part of a larger study comparing TAC monotherapy and TAC monotherapy following a high single dose of polyclonal anti-lymphocytic antibodies.

Immunosuppression protocol and clinical management

All patients had twice-daily TAC-based IS and perioperative administration of 1000 mg of hydrocortisone. Forty-nine patients had of intra-operative administration of high dose (9 mg/kg) polyclonal anti-lymphocytic serum (R-ATG®, Fresenius Biotech, Bad Homberg, Germany) under adequate anti-inflammatory coverage using hydrocortisone and acetaminophen. The first TAC oral dose (0.025 mg/kg/day) was administered at about 12 h following LT (day 0); subsequent doses were adjusted according to target blood levels of around 6 ng/ml.

All patients had similar intra- and post-transplant care. Antimicrobial agents were prescribed for 2 days adapted to the clinical status of the recipient: low-risk patients (elective and nonhemorrhagic LT) received cefazolin, whereas high-risk patients (previous prolonged hospital and intensive care unit stay, recent history of infection, urgent or hemorrhagic LT) received ceftazidime and vancomycin. Cytomegalovirus prophylaxis using ganciclovir IV (Cymevene, Roche, Basle, CH; 2×5 mg/kg/day and adapted to renal function) was used 3 weeks in IgG anti-cytomegalovirus donor positive and recipient negative pairs; oral acyclovir (Zovirax, Glaxo-Wellcome, Brentford, UK; 800 mg) was given as herpes simplex prophylaxis during 2 months.

All patients underwent careful clinical, biochemical, and histological follow-up. Histopathological scores were calculated for diagnosis of rejection and subsequent treatment. Biopsies were systematically performed on the morning of day 7 for both pathologic and TAC quantification purposes. Biopsies were read blindly by two experienced transplant pathologists and graded according to the Banff score [14,15]. Moderate to severe histological rejections were considered if the score was ≥6. Banff score <6 refer to no or mild histological rejection events. Biochemical scores were calculated based on progressive rise in total bilirubin and peripheral blood eosinophilia, absolute eosinophilia count above 600 mm^3 and progressive lowering of platelets during days 5–7 post-LT were each scored 0 to 1 [16]. The validity and validation of this score is currently under evaluation in a large series of well documented adult liver recipients. In order to avoid interference with these variables, no blood products and medications, except those mentioned above, were administered within the first post-LT week. A biochemical score of >2 was considered significant. Treatment for early rejection was administrated only in patients with clinically relevant rejection (ClR), defined by histological Banff score ≥6 with a biochemical score >2. This treatment consisted into high doses of methylprednisolone. Daily trough TAC blood concentrations were determined for dose adjustment. Additional pre-dose blood samples were taken 1, 3, 5, and 7 days after LT to measure TAC in peripheral blood mononuclear cells (PBMCs).

Tacrolimus assay

Daily TAC monitoring was performed using the chemiluminescent microparticle immunoassay (CMIA) on the ARCHITECT platform (Abbott Diagnostics, Wiesbaden, Germany). The same assay was used throughout the study period and was assessed by participation to the Tacrolimus International Proficiency Testing Scheme (TIPTS) organized by D. Holt. This method demonstrated excellent sensitivity and specificity with a close correlation with liquid chromatographic mass spectrometric methods [17].

Tacrolimus measurement in biopsies was performed by liquid chromatography/tandem mass spectrometry (LC-MS/MS) using a methodology previously described [8]. PBMCs were isolated over a Ficoll gradient, and TAC concentrations were determined by LC-MS/MS as previously published [13].

Data analysis

However achieving therapeutic efficacy is of critical importance during the initial post-transplantation period, which is generally associated to a highest risk of organ

rejection, the study was focused on the first week after LT. TAC PBMCs concentrations were compared with the severity of rejection determined by histological and biochemical grading and subsequently compared with intrahepatic TAC concentrations and mean TAC predose blood concentrations, as a marker of efficacy. Mean TAC predose concentrations were assessed from day 5 to 7, when both clinical and pharmacokinetics steady state was achieved [8].

Data are reported as the mean ± standard deviation (SD). To correlate TAC intrahepatic, PBMCs and blood TAC concentrations with the Banff scores, and TAC intrahepatic with TAC PBMCs concentrations, an unconstrained linear regression was performed, with calculation of both the coefficient of determination (R^2) and the variance analysis. ANOVA analyses were performed to analyse whether patients displayed significantly different tissue, PBMCs or mean blood TAC levels in case of clinical significant rejection at day 7. *P*-values < 0.05 were considered statistically significant. The software JMP7 (JMP software, SAS Institute, Cary, NC, USA) was used for statistical analysis.

This study was approved by the local ethical review board. All patients were enrolled after agreement and signature of an informed consent.

Results

Intrahepatic, PBMCs and blood TAC concentrations

The distribution characteristics for TAC concentrations are summarized in Table 1. No significant correlation could be observed between mean TAC blood levels and both TAC PBMCs and intrahepatic concentrations (Fig. 1). TAC PBMCs concentrations correlated with the intrahepatic TAC levels; this relationship became significant from the day 5 post-LT ($P < 0.05$) (Fig. 2 at day 7).

Table 1. Mean tacrolimus concentrations in the different compartments studied over the first week following LT: hepatic tissue, PBMCs, and blood levels. Values are expressed as mean ± standard deviation. (*n*: 90 liver transplanted patients).

Compartment	Tacrolimus concentrations (range)
Hepatic tissue day 7 (pg/mg)	91.3 ± 52.2 (10.1–294.6)
PBMCs (pg/10^6cells)	
Day 1	28.7 ± 22.2 (0–80.2)
Day 3	63.6 ± 49.8 (10.9–287.4)
Day 5	62.1 ± 40.6 (10.0–214.7)
Day 7	65.4 ± 41.8 (10.1–185.6)
Whole blood (ng/ml)	
Day 1	4.2 ± 1.6 (1.0–4.5)
Day 2	4.9 ± 2.9 (2.1–5.9)
Day 3	6.8 ± 2.1 (4.7–8.2)
Day 4	7.7 ± 6.9 (5.9–20.7)
Day 5	8.8 ± 4.8 (5.9–12.9)
Day 6	9.5 ± 6.1 (3.5–15.2)
Day 7	8.9 ± 3.0 (6.7–13.2)

Histological rejection and TAC concentrations

Thirty seven (41.1%) patients developed a moderate/severe histological rejection. These patients had significantly lower PBMCs TAC concentrations at day 3, 5, and 7, and a significantly lower intrahepatic TAC concentration at day 7 compared with the 53 patients with no/mild histological rejection. No significant differences were found between the two groups regarding blood TAC concentrations (Table 2).

Intrahepatic and PBMCs TAC concentrations at day 7 displayed a significant relationship with the Banff scores (Fig. 3a and 3b), whereas no correlation has been established regarding mean TAC blood concentrations and Banff scores (Fig. 3c).

Clinical rejection and TAC concentrations

Among the 90 patients included in this study, twelve (10.8%) developed significant clinical rejection requiring a specific treatment. These patients were characterized by significant lower day 7 TAC PBMCs concentrations (*P* 0.010) compared with patients without clinical rejection (Fig. 4a). A similar trend was observed for TAC PBMCs concentrations at day 3 (*P* 0.071), day 5 (*P* 0.056) and intrahepatic TAC concentrations at day 7 (*P* 0.059) (Table 2, Fig 4b).

Influence of intra-operative R-ATG administration

No significant difference was observed in both TAC PBMCs (*P* 0.253) and TAC hepatic concentrations (*P* 0.499) between patients receiving or not high dose R-ATG. No difference was observed regarding the incidence of both histological rejection (moderate/severe, no/mild) (*P* 0.081) and clinical significant rejection (*P* 0.792) according to ATG administration (Table 3).

Discussion

After two decades of clinical use, TAC became the major immunosuppressive agent in solid organ transplantation. Its use is characterized by considerable inter-individual variability in clinical pharmacokinetics, making TDM a necessary standard of care to ensure appropriate drug exposure and reduce side effects. Considerable effort has been put into the pharmacokinetic and -dynamic understanding of the drug. Transplant patients have

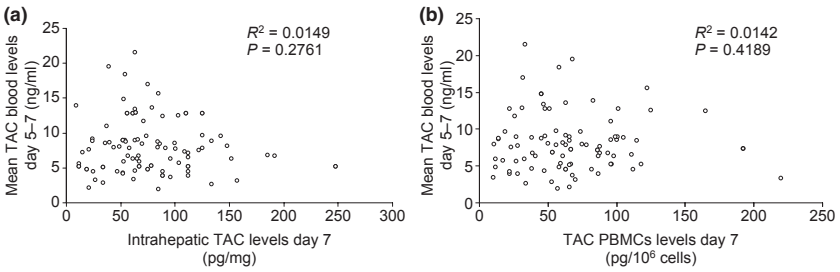

Figure 1 Correlation between mean TAC blood concentrations and both TAC intrahepatic (a) and TAC PBMCs (b) concentrations 7 days post-transplantations.

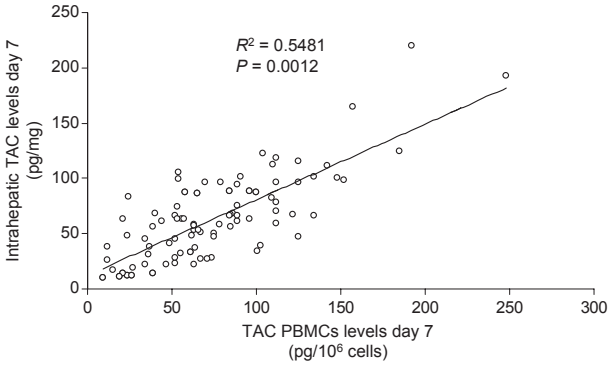

Figure 2 Correlation between TAC PBMCs and intrahepatic concentrations seven days post-transplantation. TAC PBMCs systemic exposure appears to correlate significantly with TAC intrahepatic content from day 5 post-LT.

undoubtedly benefited from this effort as blood concentration monitoring allows a rational use of TAC. Although the association between higher TAC trough concentrations and occurrence of side effects is nowadays generally recognized [5,18,19], there are more and more evidences that pre-dose concentrations might not be the best marker of drug exposure and therapeutic efficacy, stressing thereby the need to identify better biomarkers of immunosuppression [5–10,20] allowing optimizing treatment monitoring.

Two groups, including our centre, have previously demonstrated the interest of assessing the '*in situ* immunosuppression'. It has indeed been shown that hepatic tissue TAC concentration is significantly higher in patients without rejection than in patients undergoing liver rejection [8–10]. Direct quantification of TAC within the blood compartment enriched with lymphocytes, as peripheral blood mononuclear cells, appears meaningful as TAC inhibits lymphocyte calcineurin. This study investigated the impact of TAC distribution in three different compartments (blood, hepatic tissue, and PBMCs) on the incidence of both early histological and clinical significant allograft rejection. The results indicate that TAC PBMCs concentrations significantly correlated with both the development and the severity of rejection from day 3 post-LT onwards. The lower TAC PBMCs levels were associated to histological significant rejection. The study moreover confirmed our previously report, regarding the relationships between intrahepatic TAC concentrations and severity of rejection [8]. More importantly, unlike whole-blood levels, TAC PBMCs levels are associated to clinical significant rejection episode one week after transplantation. The observations made in this study empha-

Table 2. Hepatic, whole blood, and PBMCs mean (±SD) tacrolimus concentrations in patients characterized either by no/mild histological or moderate/severe histological rejection, according to their histological Banff score.

	Banff score 0–5	Banff score 6–9		
	No/mild rejection (n = 53)	Moderate/severe rejection (n = 37)	No ClR (n = 25)	ClR (n = 12)
Hepatic tissue day 7 (pg/mg)	92.8 (±57.6)	45.4 (±23.6)*	34.6 (±24.6)*	22.9 (±7.5)*
Mean whole blood day 5–7 (ng/ml)	7.3 (±4.4)	7.5 (±3.7)	6.9 (±3.9)	8.2 (±4.8)
PBMCs day 1 (pg/10^{6}cells)	24.6 (±22.8)	14.2 (±18.6)	19.3 (±12.5)	27.8 (±7.2)
PBMCs day 3 (pg/10^{6}cells)	78.6 (±36.5)	21.6 (±13.3)*	28.2 (±10.6)*	21.1 (±8.9)*
PBMCs day 5 (pg/10^{6}cells)	83.8 (±32.6)	27.6 (±12.2)*	43.9 (±17.9)*‡	19.2 (±5.3)*‡
PBMCs day 7 (pg/10^{6}cells)	90.9 (±41.2)	33.8 (±16.7)*	48.7 (±11.9)*‡	22.0 (±6.1)*‡

ClR, clinical rejection (treated rejection).
Geometric means are reported.
*P < 0.05 compared with the no/mild rejection group, ‡P < 0.05 within histological rejection group.

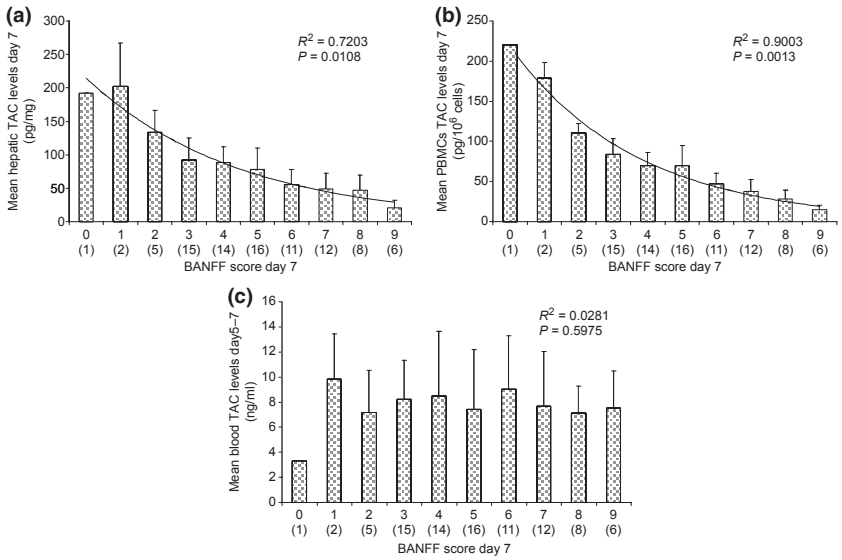

Figure 3 Correlation between either TAC intrahepatic concentrations (a) or TAC PBMCs concentrations (b) at day 7 and Banff scores determined at day 7 as histological marker of rejection. Significant first-order exponential correlation has been established in both associations. These correlations display a R^2 of 0.7203 (P = 0.0108) and 0.9003 (P = 0.0013) in intrahepatic and PBMCs respectively. No correlation has been established between blood concentration and Banff scores (c). Values are expressed as mean ± SD. Standard deviations reported correspond to interpatient variability both in PBMCs and hepatic tissue. Number of patients (n) is represented for each Banff subgroup.

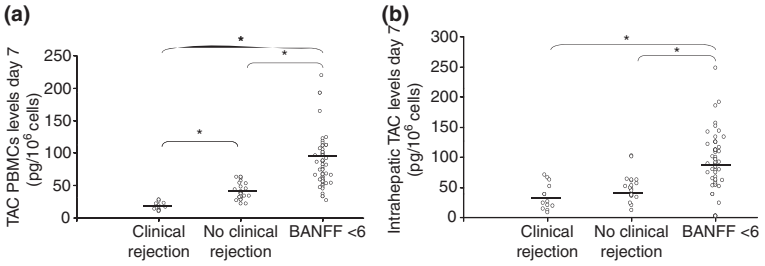

Figure 4 Comparison of both TAC PBMCs (a) and intrahepatic TAC (b) concentrations in absence or presence of relevant clinical rejection 1 week after transplantation. *P < 0.05.

Table 3. Hepatic, whole blood, and PBMCs mean (±SD) tacrolimus concentrations and clinical relevant rejection episode in patients characterized either by a pre-operative ATG administration or by the absence of such induction therapy. No difference was statistically significant.

	ATG (–) (n = 41)	ATG (+) (n = 49)
Hepatic tissue day 7 (pg/mg)	56.7 (±30.1)	71.0 (±65.2)
Whole blood (ng/ml)	6.9 (±3.4)	7.3 (±4.6)
PBMCs day 1 (pg/10^{6}cells)	36.4 (±23.9)	22.4 (±18.8)
PBMCs day 3 (pg/10^{6}cells)	70.1 (±64.5)	60.4 (±35.1)
PBMCs day 5 (pg/10^{6}cells)	65.5 (±48.4)	58.4 (±35.0)
PBMCs day 7 (pg/10^{6}cells)	68.3 (±49.7)	63.1 (±40.3)
Clinical rejection (%)	12.2	10.2
BPAR (%)	48.8	34.7

size the need for improved pharmacodynamic monitoring, and also suggest that aiming at higher TAC PBMCs exposure early after graft implantation could be of clinical interest. This strategy is of great importance since the incidence of liver allograft rejections reaches its peak around post-transplant day 7 [14,15], as demonstrated by the experimental and clinical experiences.

No significant difference has been observed in TAC PBMCs levels regardless of whether or not ATG was administered. The ability of PBMCs TAC concentrations to predict the development of clinical rejection should still be further confirmed in a larger transplant cohort.

The significant correlation found between both intrahepatic and PBMCs TAC concentrations (in contrast to whole blood levels) and the severity of rejection indicates

a non-homogeneous distribution of TAC inside hepatocytes, lymphocytes, and erythrocytes. It is also of interest to note that a high TAC PBMCs content corresponds to a higher intrahepatic concentration. This relationship could partly be explained by the activity of TAC transporter proteins at both PBMCs and hepatocytes levels. As it is known that TAC is a substrate of the P-glycoprotein (P-gp) efflux pump, cellular accumulation of TAC could be dependent on this P-gp activity, which is also mediated by environmental factors [21–24]. As these factors are identical for liver and PBMCs after transplantation, it could be speculated that the accumulation of TAC is similar in both cell types. The fact that intrahepatic TAC concentrations and TAC PBMCs levels were significantly correlated only from day 5 post-LT, could reflect an adaptation time of the hepatocytes and PBMCs P-gp activity to new environmental factors. It must be mentioned that we did not have intrahepatic data before day 7.

Numerous single nucleotide polymorphisms (SNPs) have also been described for the *ABCB1* gene affecting P-gp expression and function; some of these SNPs influence the intra-graft TAC concentrations [25]. A recent study showed that an ABCB1 polymorphism, namely G1199A SNP, is involved in the intra-lymphocytic distribution of cyclosporine [12]. Similar findings have been obtained for TAC by our group [26]. Prediction of these influences on the drug T-cell accumulation could indirectly be related to the incidence of graft rejection.

The intracellular TAC distribution (pharmacologically active exposure) must be influenced by the – potentially different (genetically-based) – P-gp expression of both donor and recipient and by similar post-transplant environmental factors. Prediction of final outcome of treatment would be difficult needing also large cohorts of patients to reach statistical significance. TAC is moreover extensively metabolized by CYP3A5, also characterized by genetic polymorphism, directly influencing dose requirements and trough blood TAC levels [27–29]. The impact of CYP3A5 expression on TAC intra-lymphocytic distribution is unclear but could be an additional variable to take into account. Furthermore, the expression of both CYP3A5 and P-gp is regulated by the pregnane X receptor (PXR), a key regulator in drug metabolism and efflux. This polymorphic receptor expressed in lymphocytes and other tissues could also influence intra-lymphocyte TAC levels by acting on the expression of P-gp and/or CYP3A5 in the recipient and the transplanted organ [30–32].

Our study demonstrates the interest of TAC PBMCs level as a marker of efficacy early after LT. Although TAC PBMCs concentrations cannot yet be considered as a routine test, its clinical application appears easier than the tissue drug measurement, which requires invasive biopsies. The clinical relevance of this approach, associated to TAC blood concentrations, still needs to be evaluated in larger prospective trials, where genetic aspects of the donor and recipient should be considered, as well as the different immunosuppression protocols (drug combinations, delayed TAC introduction,…). The originality of this study is related to the fact that a monotherapy was used by purpose in order to eliminate possible interactions between different immunosuppressive drugs, and to identify more easily the interest of intracellular drug concentrations as markers of rejection, over histological and/or TAC blood concentrations. From our experience it is clear that the transplant recipient should be followed in a very individualized way, integrating clinical examination, biochemical evolution, pathology report as well as pharmacodynamics and pharmacokinetics of the immunosuppressive drug. This integrated approach should be considered as a powerful diagnostic tool, also able to prevent unnecessary and potentially harmful anti-rejection treatment. The reported results shed new lights on an original and more tailored approach to optimize immunosuppression after liver transplantation.

Authorship

AC, JL, VH, and PW: wrote the paper. AC, JL, DL, JR, VH, and PW: designed the study. AC: performed the study and analyzed the data.

Funding

This study has been supported in part by a Junior Clinical grant from the European Society for Organ Transplantation (ESOT), and by a national research grant from FRSM (FRSM 3.4.586.08 FCT).

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