Pharmacodynamic monitoring of calcineurin inhibition therapy : investigation of the calcineurin activity marker

Rossum, H.H. van

Chapter 8

Summary and General Discussion

Chapter 8: Summary and general discussion

Over the last decades, restoration of renal function by renal allograft transplantation has evolved into the preferred treatment option for patients with end stage renal disease. The introduction of the calcineurin inhibitors (CNI) cyclosporine (CsA) and tacrolimus (TRL) have significantly contributed to this success. Both CNIs allowed successful suppression of the immunological response and rejection of the allograft by the recipient(1). Unfortunately considerable adverse drug effects are observed in patients treated with CNIs, including nephrotoxicity, hypertension and diabetes mellitus(2-4). This, together with the large inter-individual variation in pharmacokinetics of both drugs necessitates therapeutic drug monitoring (TDM) to control drug exposure(5). Nowadays, TDM of CNI is routinely performed by drug concentration measurement in blood which has led to an improved clinical performance of these drugs. Though acute rejection episodes have been significantly reduced, the incidence of acute allograft rejection episodes is still 10-20% within first year after transplantation(6). In addition, lack of selectivity and over-immunosuppression, at least partly caused by CNIs, hampers long-term allograft and patient survival. CNI withdrawal and avoidance protocols have been investigated with varying results. Early removal of CNIs from the immunosuppressive protocols continues to be problematic mainly due to the occurrence of late acute rejection(7,8). Minimization of CNIs in the immunosuppressive protocols early after transplantation was recently found to be equally effective as compared to the “old standards” with higher CNI target concentrations(9).

Another strategy to improve clinical immunesuppresion early after transplantation is improved monitoring to control CNI therapy. TDM for CNI treatment is mandatory since there is a clear relation between drug exposure and incidence of acute rejection and drug toxicity(10,11). Improvement of CNI therapy by better monitoring is an interesting strategy, since in current daily practice TDM is performed under suboptimal monitoring conditions. This includes TDM by through drug concentrations (C0) and non-standardized sampling protocols. Next to advanced pharmacokinetic monitoring, such as estimated AUC monitoring, the development of pharmacodynamic (PD) markers could theoretically contribute to improve CNI therapy. Pharmacodynamic monitoring strategies, however, are still in an experimental phase and have not proven clinical benefit yet. They carry the theoretical advantage of monitoring the true effectiveness of immunosuppressive therapy including CNIs. In addition, pharmacological and immunological drug-drug interaction can be detected. This led us to investigate PD monitoring as potential

tool to guide drug dosing. We choose CN activity as PD marker for monitoring and in this thesis, the analytical aspects, fundamental characteristics and insights in clinical usefulness of CN activity measurement as a PD marker for CNI were investigated.

(Bio) analytical aspects of CN enzyme activity measurement

As starting point to research PD monitoring of CN inhibition therapy a CN enzyme activity assay was developed (**Chapter 2**). CN activity was determined in the leukocyte fraction isolated from peripheral blood. CN activity was determined by phosphate quantification after dephosphorylation of the phosphorylated RII substrate. Phosphate concentration was determined using the malachite green phosphate reagent. To adjust for background absorbance, a blank measurement without substrate was included. Ascorbic acid, NP-40 and DTT were titrated in the lysis buffer that resulted in a significant increase in CN activity and conservation of CN activity during sample storage. This was hypothized to be caused by prevention of CN oxidation that is known to be enzymatically inactivated by oxidation(12,13). Furthermore CN inhibition by both TRL and CsA was found *in vitro* as well as in renal allograft recipients treated with either TRL or CsA.

In **Chapter 3** we investigated the relation between CN inhibition, inhibition of T cell cytokine excretion and their relation at therapeutic CsA concentrations. Blood from healthy volunteers incubated with relevant high range CsA concentrations (2500 µg/L) showed only a minor increase in CN inhibition when CsA was added in excess (25000 µg/L). In addition, no increase in cytokine excretion after CD3/CD28 stimulation was observed. This indicated that in the system used, at 2500 µg/L, CsA approaches immunosuppressive saturation. At relevant target C0/C2 levels early and late after renal transplantation (100, 250, 600 and 1700 µg/L) every condition represented a distinct immunosuppressive state. In addition after an initial CN inhibition threshold, small increases in CN inhibition resulted in relatively large changes in T cell cytokine excretion. No cyclophilin limitation at 2500 µg/L CsA could be detected. These results showed that CN activity could act as a relevant marker to monitor CsA based immunosuppression.

As a follow-up, we investigated whether the leukocyte fraction is a representative matrix for monitoring CsA therapy by CN activity measurement in renal allograft recipients. This is described in **Chapter 4**. Large inter-individual variation in CN activity was observed in renal allograft recipients receiving CsA. In addition, large variation in sample composition was observed when parallel to CN activity,

leukocyte concentrations of granulocytes, monocytes and lymphocyte subsets including T cells B cells and NK cells in peripheral blood were determined. Next, cell specific CN activities and CsA inhibition curves were determined and revealed cellular differences in CN activity. Similar cellular differences have been observed previously(14). No clear deviations in CsA inhibition profiles for the different leukocyte subsets were observed as has been described before(15). Altogether, these results could indicate that sample composition, at least partially, determines CN activity measurement outcome. When applying CN activity monitoring in clinical practice CN activity could therefore be biased by sample composition. Interestingly, the sample composition effect could also be relevant for other PD markers used such as mRNA transcripts or cytokines excretion markers. Here T cell specific mRNA transcripts isolated from the mononuclear cell fraction are used as readout and expressed against reference transcripts available in all mononuclear cells(16-18). Variation in relative T cell concentration between and within individuals could therefore confound these markers in a similar way. An interesting next step would be to monitor specifically in the target compartment(s) of CNI; the T cells. T cell specific readout assays could eliminate the compositional effects on measurement outcome and in addition, monitor in the immunological relevant cells(19,20).

To anticipate on the suggested higher specificity of T cell specific pharmacodynamic assays, we have developed a T cell specific CN assay for monitoring CsA therapy (**Chapter 6**). In this protocol T cells were isolated from peripheral blood using paramagnetic beads coated with anti-CD3 antibodies. T cells isolates from peripheral blood were highly pure 95% ± 1.5% and showed to be a representative sample of whole blood T cells, since no differences in CD4+ and CD8+ subsets were found. When coupled to the CN activity assay protocol, adequate analytical performance was observed and no washing out of CsA during sample preparation. In addition, in 6 renal allograft recipients receiving CsA therapy, inhibition of T cell specific CN activity was observed. In future studies it would be very interesting to investigate the relevance of the T cell specific calcineurin activity marker as tool for monitoring CsA therapy. Measurement of other intracellular T cell markers such as protein concentrations or their activities, drug concentrations and metabolic compounds could be very relevant markers for a large variety of diagnostic fields.

Fundamental properties of PD strategies

To gain insights in the current status of PD monitoring for CNI therapy, a review of

recent literature on this subject was performed (**Chapter 5**). Two different types of PD monitoring strategies have been investigated. The first type monitors enzyme activity and the second cellular responsiveness after *in vitro* stimulated immunological responses. CN activity has been measured in various cellular fractions such as whole blood, leukocytes and peripheral blood mononuclear cells (PBMC) and is usually performed in conditions of co-reagent excess. It is therefore more appropriate to refer to as CN capacity measurement. For immunological strategies simulation of immunological responses is performed by mitogen or CD3/CD28-directed T cell activation. Immune responsiveness has been investigated at several levels such as mRNA transcripts, (intracellular) concentration/excretion of cytokines, expression of surface activation markers and cell proliferation. So far, clear relations of such markers with drug concentrations have been observed, but no definite clinical benefits have been reported(19,21,22). The large inter-individual variation of pharmacodynamic marker outcome could not been explained by clinical end-points such as acute rejection. Better insight in the large inter-individual variation is relevant to understand and further develop new, more accurate markers. Strategies including pharmacodynamic parameters have proven to be valuable in studying pharmacological drug-drug interactions of combinational immunosuppressive therapy applied in allograft recipients and visualizing *in vivo* immunosuppression(23-26).

We have used pharmacodynamic strategies to investigate the drug-drug interaction between TRL and the mTOR inhibitors sirolimus (SRL) and everolimus (EVL) (**Chapter 7**). Combinational therapies including mTOR inhibitors are increasingly applied in allograft recipients. However, based on the pharmacological mechanism of mTOR inhibition by the mTOR inhibitors and CN inhibition by TRL, pharmacological interactions can be expected. Both drugs bind to FK-binding proteins and complexes of either FK-binding protein with TRL or mTOR inhibitor selectively inhibit CN and mTOR respectively(27-29). We have tested the effect of the mTOR inhibitors SRL and EVL on TRL based CN inhibition in PBMC cell cultures. First, no CN inhibition was observed for both SRL and EVL. Next combinations of mTOR inhibitor with TRL led to increased TRL IC_{50} values for CN activity as well as IL2 excretion after mitogen stimulation. This was reproduced in 10 healthy volunteers. No antagonism was observed for CsA, which does not require FKBPs for CN inhibition. When FKBP12 was added to cell lysates TRL antagonism by SRL and EVL could be reversed. Altogether, these results indicate that competition for FKBP binding sites mediates the antagonism. Since all

experiments have been performed in culture conditions, the clinical relevance of the antagonism remains unknown. Due to the presence of irrelevant binding sites in whole blood that are absent in culture medium extrapolation of culture concentrations is required, as has been demonstrated for CsA(30). It would be very relevant to investigate whether this antagonism occurs under relevant *in vivo* blood concentrations of mTOR inhibitors and TRL.

Overall conclusions and future perspectives

This thesis shows that pharmacodynamic monitoring strategies, including CN activity, are very helpful in describing immunosuppressive responses and interactions. Especially in well controlled *in vitro* experimental set-ups these assays can be used to investigate drug properties and interactions. CN activity was very useful to illustrate the pharmacological and immunological interaction of TRL with the mTOR inhibitors SRL and EVL. This rate limiting interaction is mediated via competition for the relevant chaperone for both drug classes. A relevant follow-up would be to investigate the clinical relevance of this interaction.

Unfortunately, the pharmacodynamic monitoring strategies are not ready yet for clinical use as marker to guide drug dosing. For the final transition from investigative tools to relevant biomarkers, insight in the analytical and biological variables appears to be of key importance. We have illustrated the importance of sample/matrix choice. Confounding of measurement outcome by sample composition should be avoided or at least adjusted for. Specific measurement in the relevant pharmacological and immunological target compartments of CNI to evade from these methodological confounders.

In this respect we have developed a T cell specific CN assay that showed acceptable analytical performance. T cell specificity could also contribute to improved performance of other pharmacodynamic markers for monitoring CNI therapy such as T cell specific cytokines, mRNA and cell surface T cell activation markers. This could be performed by for instance T cell specific stimulation and includes the activation of T cells by targeting specific T cell epitopes e.g. CD3 and CD28. In such assays, optimization of the time interval should prevent secondary activation of other cells present due to cytokines produced by activated T cells. Finally, T cell specific assays to asses T cell specific CN activity need to be validated as a marker for monitoring CsA therapy in a large cohort of patients treated with CNIs in order to proof their clinical relevance.

This thesis has demonstrated that the anticipation on analytical confounders, such

as sample choice, is crucial for the development of CN activity as a marker that has the intrinsic property to visualize CNI pharmacodynamics.

References

1. Sayegh MH, Carpenter CB. Transplantation 50 years later - Progress, challenges, and promises. New England Journal of Medicine 2004;351:2761-6.
2. Curtis JJ. Hypertensinogenic mechanism of the calcineurin inhibitors. Current Hypertension Reports 2002;4:377-80.
3. Heisel O, Heisel R, Balshaw R, Keown P. New onset diabetes mellitus in patients receiving calcineurin inhibitors: A systematic review and meta-analysis. American Journal of Transplantation 2004;4:583-95.
4. Naesens M, Kuypers DRJ, Sarwal M. Calcineurin Inhibitor Nephrotoxicity. Clinical Journal of the American Society of Nephrology 2009;4:481-508.
5. Budde K, Glander P. Pharmacokinetic principles of immunosuppressive drugs. Annals of Transplantation 2008;13:5-10.
6. Meier-Kriesche HU, Schold JD, Srinivas TR, Kaplan B. Lack of improvement in renal allograft survival despite a marked decrease in acute rejection rates over the most recent era. American Journal of Transplantation 2004;4:378-83.
7. Cravedi P, Ruggenenti P, Remuzzi G. Sirolimus to replace calcineurin inhibitors? Too early yet. Lancet 2009;373:1235-6.
8. Srinivas TR, Schold JD, Guerra G, Eagan A, Bucci CM, Meier-Kriesche HU. Mycophenolate mofetil/sirolimus compared to other common immunosuppressive regimens in kidney transplantation. American Journal of Transplantation 2007;7:586-94.
9. Ekberg H, Tedesco-Silva H, Demirbas A, Vitko S, Nashan B, Gurkan A et al. Reduced exposure to calcineurin inhibitors in renal transplantation. New England Journal of Medicine 2007;357:2562-75.
10. Mahalati K, Belitsky P, Sketris I, West K, Panek R. Neoral monitoring by simplified sparse sampling area under the concentration-time curve - Its relationship to acute rejection and cyclosporine nephrotoxicity early after kidney transplantation. Transplantation 1999;68:55-62.
11. Mahalati K, Belitsky P, West K, Kiberd B, Fraser A, Sketris I et al. Approaching the therapeutic window for cyclosporine in kidney transplantation: A prospective study. Journal of the American Society of Nephrology 2001;12:828-33.
12. Ghosh MC, Wang X, Li S, Klee C. Regulation of calcineurin by oxidative stress. Methods Enzymol 2003;366:289-304.
13. Wang XT, Culotta VC, Klee CB. Superoxide dismutase protects calcineurin from inactivation. Nature 1996;383:434-7.
14. Blanchet B, Hulin A, Ghaleb B, Giraudier S, Jouault H, Astier A. Distribution of calcineurin activity in blood cell fractions and impact of tacrolimus inhibition. Fundamental & Clinical Pharmacology 2006;20:137-44.
15. Kung L, Batiuk TD, Palomo-Pinon S, Noujaim J, Helms LM, Halloran PF. Tissue distribution of calcineurin and its sensitivity to inhibition by cyclosporine. Am J Transplant 2001;1:325-33.
16. Giese T, Zeier M, Meuer S. Analysis of NFAT-regulated gene expression in vivo: a novel perspective for optimal individualized doses of calcineurin inhibitors. Nephrology Dialysis Transplantation 2004;19:55-60.
17. Hartel C, Fricke L, Schumacher N, Kirchner H, Muller-Steinhardt M. Delayed cytokine mRNA expression kinetics after T-lymphocyte costimulation: A quantitative measure of the efficacy of cyclosporin A-based immunosuppression. Clinical Chemistry 2002;48:2225-31.
18. Hartel C, Schumacher N, Fricke L, Ebel B, Kirchner H, Muller-Steinhardt M. Sensitivity of whole-blood T lymphocytes in individual patients to tacrolimus (FK 506): Impact of interleukin-2 mRNA

expression as surrogate measure of immunosuppressive effect. Clinical Chemistry 2004;50:141-51.

19. Jorgensen KA, Koefoed-Nielsen PB, Karamperis N. Calcineurin phosphatase activity and immunosuppression. A review on the role of calcineurin phosphatase activity and the immunosuppressive effect of cyclosporin A and tacrolimus. Scand J Immunol 2003;57:93-8.
20. van Rossum HH, Romijn FPHT, Sellar KJ, Smit NPM, van der Boog PJM, de Fijter JW, van Pelt J. Variation in leukocyte subset concentrations affects calcineurin activity measurement: Implications for pharmacodynamic monitoring strategies. Clinical Chemistry 2008;54:517-24.
21. Sommerer C, Giese T, Meuer S, Zeier M. Pharmacodynamic monitoring of calcineurin inhibitor therapy: Is there a clinical benefit? Nephrol Dial Transplant 2008;epub/ahead of print.
22. Yano I. Pharmacodynamic monitoring of calcineurin phosphatase activity in transplant recipients treated with calcineurin inhibitors. Drug Metab Pharmacokinetics 2008;23:150-7.
23. Barten MJ, Streit F, Boeger M, Dhein S, Tarnok A, Shipkova M et al. Synergistic effects of sirolimus with cyclosporine and tacrolimus: Analysis of immunosuppression on lymphocyte proliferation and activation in rat whole blood. Transplantation 2004;77:1154-62.
24. Fukudo M, Yano I, Masuda S, Fukatsu S, Katsura T, Ogura Y et al. Pharmacodynamic analysis of tacrolimus and cyclosporine in living-donor liver transplant patients. Clinical Pharmacology & Therapeutics 2005;78:168-81.
25. Giese T, Zeier M, Schemmer P, Uhl W, Schoels M, Dengler T et al. Monitoring of NFAT-REGULATED gene expression in the peripheral blood of allograft recipients: A novel perspective toward individually optimized drug doses of cyclosporine A. Transplantation 2004;77:339-44.
26. Halloran PF, Helms LM, Kung L, Noujaim J. The temporal profile of calcineurin inhibition by cyclosporine in vivo. Transplantation 1999;68:1356-61.
27. Dumont FJ, Staruch MJ, Koprak SL, Melino MR, Sigal NH. Distinct Mechanisms of Suppression of Murine T-Cell Activation by the Related Macrolides Fk-506 and Rapamycin. Journal of Immunology 1990;144:251-8.
28. Dumont FJ, Melino MR, Staruch MJ, Koprak SL, Fischer PA, Sigal NH. The Immunosuppressive Macrolides Fk-506 and Rapamycin Act As Reciprocal Antagonists in Murine T-Cells. Journal of Immunology 1990;144:1418-24.
29. Schuler W, Sedrani R, Cottens S, Haberlin B, Schulz M, Schuurman HJ et al. SDZ RAD, a new rapamycin derivative - Pharmacological properties in vitro and in vivo. Transplantation 1997;64:36-42.
30. Batiuk TD, Pazderka F, Enns J, DeCastro L, Halloran PF. Cyclosporine inhibition of leukocyte calcineurin is much less in whole blood than in culture medium. Transplantation 1996;61:158-61.