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ORIGINAL ARTICLE

A retrospective study of the prognostic impact of cytokine secretion in mixed lymphocyte culture on long-term graft function following allogeneic renal transplantation

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Summary

We have previously shown that *in vitro* measurement of cytokine production prior to renal transplantation can provide predictive information on the risk of acute rejection. Our earlier studies demonstrated that patients who secreted high levels of interferon-gamma (IFN-γ) in OKT3-stimulated or mixed lymphocyte culture had a significantly increased risk of acute rejection compared with patients who secreted lower levels. In this study, we performed a retrospective analysis of the same cohort of patients in order to determine the prognostic value of cytokine profiles and other variables on long-term graft function. Our results show that high levels of IFN-γ in pretransplant mixed lymphocyte culture are a highly significant predictor of poorer creatinine levels at 18, 24 and 36 months post-transplant.

Introduction

Although it is accepted that human leucocyte antigen (HLA) compatibility is associated with a decreased incidence of acute allograft rejection and improved long-term survival following renal transplantation [1,2], other immunological molecules also play an important role in transplant outcome. Cytokines are important mediators of the regulation of immune responses and alloreactivity and we have previously demonstrated, *in vitro*, significant inter-individual variations in mitogen-stimulated cytokine production in both normal individuals [3] and patients [4] prior to renal transplantation. In a subsequent study, we measured cytokine secretion in patient-versus-donor mixed lymphocyte culture (MLC) in a cohort of 57 renal patients prior to transplantation. Secretion of interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-10 (IL-10) and interferon-gamma (IFN-γ) protein were measured. We demonstrated that high levels of pretransplant IFN-γ and IL-10 in MLC, together with HLA mismatching (especially HLA-DR) and female donor sex, were significantly associated with rejection [5]. However, multivariate analysis revealed that the greatest risk of rejection was associated with a combination of high IL-10 secretion in MLC and mismatching for at least four HLA ($R^2 = 25.5$, $P = 0.003$) [5]. Recently it has been shown that graft function at 6 and 12 months is a more powerful predictor of long-term outcome [6] than the occurrence of acute rejection. We therefore performed a retrospective study to examine the influence of pretransplant cytokine profiles on creatinine levels as a surrogate marker of graft function.

Patients and methods

Patients

Thirty-three patients who received a cadaveric renal transplant between 1997 and 1999 at Derriford Hospital, Plymouth and who were included in our earlier cohort, were studied. All patients received standard triple immunosuppressive therapy with ciclosporin, azathioprine and prednisolone.

Assessment of long-term graft function

Graft function was assessed by serial measurement of serum creatinine levels.

Preparation and storage of cells

This was performed as described previously [5]. Briefly, recipient peripheral blood and donor spleen were collected immediately prior to transplantation and before any immunosuppressive treatment had been administered. Mononuclear cells from the peripheral blood and spleen were isolated by density gradient centrifugation and cryopreserved until required.

Mixed lymphocyte cultures

This was performed as described previously [5]. Briefly, the cryopreserved cells were thawed, washed and the concentrations adjusted to 1×10^6 cells/ml. Donor cells were irradiated at 30 Gy. Patient-versus-donor MLCs were subsequently set up in 24 well tissue culture plates and incubated for the calculated optimal time for each cytokine at 37 °C in 5% CO_2 (72 h for IL-2 and IL-10, 96 h for IL-4 and IFN-γ and 120 h for IL-6). Following incubation, cell-free supernatants were harvested and stored at −20 °C until required for cytokine analysis.

Cytokine assays

These were performed as described previously [5]. Briefly, the MLC supernatants were thawed and assayed in duplicate by commercial enzyme-linked immunosorbent assay kits for IL-2 (R&D Systems, Oxon, UK), IL-4, IL-6, IL-10 and IFN-γ (Eurogenetics Ltd, Hampton, UK) according to the manufacturers instructions. The plates were read at 450 nm and the samples calculated using a Dias Microplate Reader (Dynatech Laboratories, Sussex, UK).

Statistical analysis

The results of the cytokine analyses together with other relevant variables; i.e. HLA matching, recipient creatinine, recipient and donor sex, recipient and donor cytomegalovirus (CMV) status, recipient and donor age, numbers of previous transplants, primary graft function, donor creatinine, cold and warm ischaemia time, numbers of rejection episodes and donor cause of death were analysed using both univariate and multivariate (multiple regression) statistical methods. As the creatinine values were not normally distributed, natural logarithms were taken of these values at each time so that parametric statistical methods could be used. The log creatinine values were assessed for normality using Shapiro–Wilks tests. Natural logarithms were also taken of the IFN-γ and IL-10 values. An outlier was omitted from the analyses at 24, 36 and 60 months. The reason for this was that it was subsequently found that this patient had been infected with a polyoma virus that could cause allograft dysfunction and graft loss. Independent samples *t*-tests, one-factor analysis of variance (ANOVA) and Pearson correlation coefficients were used, as appropriate, in the univariate analysis to explore the relationship between the (log) creatinine values and the above variables.

Multiple linear regressions (forward step-wise) were used to model the (log) creatinine values at all time points from 6 to 60 months on those variables for which $P \leq 0.1$ in the univariate analyses. The number of rejection episodes was not included in the multiple regressions as significant correlations between cytokine secretion and acute rejection had previously been demonstrated and the purpose of this study was to determine whether an association existed with long-term graft function. The strength of the association with acute rejection was such that, had it been included, it would have masked the other variables. The final multiple regression models included only those variables that were significant at $P < 0.05$.

Results

Univariate analyses

The results of the univariate analyses are shown in Table 1. *P*-values are shown where these are less than or equal to 0.1. Blank entries in the table indicate $P > 0.1$. The analysis revealed that there were no significant findings ($P < 0.1$) at any time with respect to recipient age, cold ischaemia time, HLA A or B mismatching. The mean (log) creatinine levels were lower for female recipients; female donors; negative recipient CMV status; negative donor CMV status; no previous transplants; primary graft function; low log pre IL-10; low log pre IFN-γ; younger donor age; lower donor creatinine; fewer rejection episodes; donor cause of death head injury or tumour (although only one of latter); two antigen HLA DR mismatch. Figure 1 shows the relationship between (log) serum creatinine at 24 months and pretransplant log IFN-γ.

Table 1. Results of univariate analyses.

	(Log) Creatinine (months)									
Variable	6	12	18	24	30	36	42	48	54	60
Recipient sex*	0.013	0.06	0.05		0.023	0.096	0.053	0.06		
Donor sex*	0.06	0.011			0.026	0.044	0.006	0.028	0.016	0.07
Recipient CMV*	0.06	0.013	0.037	0.005	0.006	0.007		0.035	0.07	0.021
Donor CMV*					0.021		0.055	0.065	0.07	
Number of transplants*		0.07	0.029			0.075				
Primary graft function*					0.06		0.032	0.09	0.06	0.09
Log pre IL-10†	0.06		0.04	0.021		0.1				0.06
Log pre IFN-γ†	0.06	0.033	0.004	<0.001		0.002				
Donor age†	0.015	0.031		0.055	0.065	0.041	0.013	0.008	0.009	0.011
Donor creatinine†										0.038
Warm Ischaemia time†	0.09									
Rejection episodes†	<0.001	0.004	<0.001	0.005	0.029	0.001	0.032	0.031		
Donor cause of death‡				0.07						
MMDR‡						0.08				

CMV, cytomegalovirus; IL, interleukin; IFN-γ, interferon-gamma.
P-values are shown where these are less than or equal to 0.1.
*Independent samples *t*-test.
†Pearson correlation.
‡One-factor ANOVA.

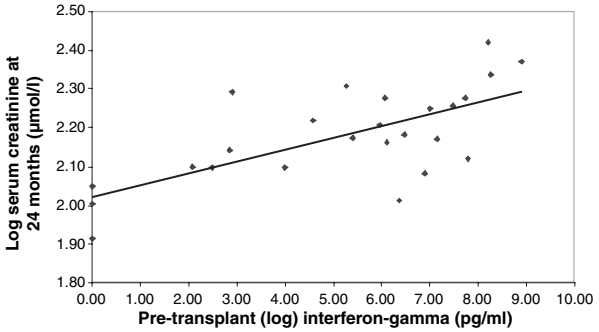

Figure 1 Relationship between (log) serum creatinine at 24 months and pretransplant (log) interferon-gamma.

Multiple regression analyses

The results of multiple regression analysis using forward selection (step-wise) on all those variables that were significant at $P \leq 0.1$ in the univariate analyses are shown in Table 2. *P*-values of less than 0.05 are given. Variables not included in the table were not significant predictors at any of the times. The multiple regression models are reasonably good predictors of log creatinine, particularly at 18, 24 and 42 months when the R^2 value is over 50%. Some patterns and trends are apparent. IFN-γ is a significant predictor of (log) creatinine at 18, 24 and 36 months only. IL-10 is not a significant predictor at any time point in the multiple regressions. Recipient CMV status is a significant predictor of (log) creatinine at most of the earlier times, whereas donor CMV status is significant at the later times. Donor age is a significant predictor at 6 months and at 42, 48, 54 and 60 months. Further details of the multiple regression models at 18, 24 and 36 months are given in Table 3. At these times IFN-γ and recipient CMV status were the only significant predictors of (log) creatinine. The models at the three times are very similar. For example, in month 18 the estimated β means that, on average, an increase of 1 in log INF-γ would lead to an increase of 0.056 in log creatinine.

Discussion

We have demonstrated, for the first time, that pretransplant measurement of IFN-γ in patient-versus-donor MLC is a significant predictor of long-term graft function, as assessed by creatinine, at 18, 24 and 36 months post-transplant. Our analyses did show that acute rejection remained the strongest predictor of long-term graft function, but the strength of the association was such that, had it been included, it would have masked the other variables. In addition our previous studies on the same cohort of patents demonstrated a significant correlation between IFN-γ and rejection [5]. Our findings differ from the recent study of Hariharan *et al.* [6], which found that 1-year creatinine was the best predictor of long-term graft function. We also found that recipient CMV status correlates with early graft function whereas donor CMV status and donor age predicts graft function at later time points. Although in general, CMV reactivation occurs between

Table 2. Results of multiple regression analysis using forward selection (step-wise) on all those variables that were significant at $P \leq 0.1$ in the univariate analyses.

	(Log) Creatinine at (months)									
Variable	6	12	18	24	30	36	42	48	54	60
Recipient CMV	NS	0.021	0.026	0.007	0.010	0.014	NS	NS	NS	NS
Recipient sex	0.014	NS	NS	NS	NS	NS	NS	NS	NS	NS
Donor CMV	NS	NS	NS	NS	0.033	NS	0.019	0.020	0.042	NS
Log pre IFN	NS	NS	0.003	<0.001	NS	0.005	NS	NS	NS	NS
Donor sex	NS	0.017	NS	NS	NS	NS	0.020	NS	NS	NS
Donor age	0.016	NS	NS	NS	NS	NS	0.012	0.003	0.006	0.007
Donor creatinine	NS	NS	NS	NS	NS	NS	NS	NS	NS	0.023
R^2 (%)	35.7	35.5	50.2	60.9	41.9	45.7	55.5	36.8	36.6	41.1

CMV, cytomegalovirus; IFN, interferon.
NS, not significant ($P \geq 0.05$).
P-values of less than 0.05 are given, showing *P*-values ≤ 0.05 and R^2 values (%).

Table 3. Estimated regression parameters and their 95% confidence intervals for the multiple regression models at 18, 24 and 36 months. At these times IFN-γ and recipient CMV status were the only significant predictors of (log) creatinine.

	Estimated regression parameters	95% CIs
Month 18		
Log IFN-γ	0.056	0.022–0.091
Recipient CMV	0.206	0.028–0.384
Month 24		
Log IFN-γ	0.060	0.031–0.090
Recipient CMV	0.226	0.067–0.384
Month 36		
Log IFN-γ	0.055	0.019–0.091
Recipient CMV	0.243	0.054–0.432

CMV, cytomegalovirus; IFN-γ, interferon-gamma.

one and four months post-transplant, regardless of whether this is reactivation of latent virus in the patient or acquired from the donor, cases occurring many months later have been documented [7]. In addition, the size of this study makes the significance of these findings unclear.

The IFN-γ is a pleiotropic cytokine involved in the regulation of nearly all phases of immune and inflammatory responses, including the activation, growth and differentiation of T cells, B cells, NK cells and other cell types such as endothelial cells and fibroblasts. It enhances HLA expression on antigen-presenting cells and is a hallmark of T helper 1(Th1) differentiation [8]. In recent years it has become apparent that large variations in the production of cytokine protein exist between individuals. In the case of IFN-γ, we have previously demonstrated significant inter-individual variations in the levels of *in vitro* IFN-γ production in mitogen-stimulated culture and MLC in both normal individuals and patients prior to renal transplantation [3–5]. These studies have shown that the ability to secrete high levels of IFN-γ, is part of a general phenomenon of 'high responsiveness' that can be triggered by a mitogen such as OKT3, or PHA or by histocompatibility differences, particularly HLA class II antigens, in MLC.

We have also demonstrated that transplant patients who secrete increased levels of IFN-γ in both mitogen-stimulated culture and MLC are more likely to reject their kidneys than patients who secrete lower levels [4,5]. Similarly, Sadeghi *et al.* [9] have demonstrated that patients with high pretransplant serum levels of IFN-γ are at risk of early acute rejection while Heeger *et al.* [10] have shown that patients with high pretransplant frequencies of donor-specific, IFN-γ producing lymphocytes are at risk of acute rejection. Whether these phenomena explain the observed association between IFN-γ and graft function is not yet clear.

Interestingly enough, no association was demonstrated between pretransplant secretion of IL-10 in MLC and long-term graft function. This is in accordance with data from Asderakis *et al.* [11] showing a long-term protective effect in patients with a high IL-10 secretor genotype. However, this was in contrast to our previous work [5] and that of others [12–14] showing an association between high levels of IL-10 or high numbers of IL-10 secreting lymphocytes and acute rejection after renal transplantation. One explantation for this discrepancy is the possibility that high IL-10 levels in the early post-transplant period are simply a surrogate marker for other more important Th1 cytokines, such as IFN-γ. Our own unpublished observations suggest that some individuals are 'high secretors' for both Th1 and Th2 cytokines.

An important issue in transplantation is that of genetic regulation of cytokine synthesis. Although a number of groups have demonstrated significant correlations between a number of cytokine gene polymorphisms and organ allograft rejection [15] others have failed to show any correlation between cytokine gene polymorphisms and protein production [16]. As far as the genetic control of IFN-γ protein is concerned, Pravica *et al.* [17] demonstrated a relationship between IFN-γ genetic polymorphisms and the *in vitro* production of IFN-γ. Our group, however, was unable to demonstrate such a correlation, nor indeed any association between IFN-γ gene polymorphisms and acute rejection [18]. In contrast, Hutchinson's group have shown that IFN-γ polymorphisms can predict both acute graft rejection [11] and graft function [11,19]. The latter is in accordance with the data that we present in this paper. The conflicting data on genetic influence on cytokine profiles do not alter the observations presented here that suggest IFN-γ has a significant influence on graft function in the midterm and further work is required to elucidate the potential mechanisms of this interaction.

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