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The TNF-α system after successful living-related kidney transplantation

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Abstract The TNF-α system is thought to play a central role in the reduced immunity of haemodialysis patients. The imbalance between the high levels of soluble TNF receptors R1 and R2 and the low levels of immunoactive TNF-α results in an increased TNF-α buffering capacity leading to reduced immune responses. Apart from impaired renal clearance of the receptors, inefficient TNF-α production as a result of the uraemia may also contribute to the imbalance between this cytokine and its receptors. In patients receiving a living-related kidney transplant, renal function is nearly normalized in a very short period. This restoration of renal function may result in a state of better immunocompetence, either as a result of improved clearance of the receptors or as a result of reversal of the uraemic state. To differentiate between these two possibilities, we measured TNF-α protein, mRNA and the soluble TNF receptors R1 and R2 before and after successful renal transplantation. TNF-α mRNA was not affected by transplantation, indicating constant TNF-α production. The imbalance in the TNF-α system was markedly improved after transplantation, although normal values of the soluble receptors were not reached. One year after transplantation in stable kidney transplant recipients there was still an imbalance in the TNF-α system caused by persistently elevated levels of the soluble TNF-receptors. These results suggest that even after successful kidney transplantation the TNF-α system remains activated. However, despite immunosuppressive therapy, recipients of a living-related kidney do have a better balanced TNF-α system compared to haemodialysis patients.

Key words TNF-α · Soluble TNF receptors · Living-related · Kidney transplantation

Introduction

Patients with renal insufficiency suffer from a high incidence of infections and malignancies [1, 2]. This may be a result of generalized immunodeficiency owing to an imbalance of the defence mechanisms in which especially TNF-α and its soluble receptors are thought to play an important role [3–5].

Previously we have found increased levels of soluble TNF receptors (sTNF-R) in peripheral blood of patients on haemodialysis and peritoneal dialysis and in patients with preterminal end-stage renal failure [6]. Using reverse transcriptase polymerase chain reaction (RT-PCR) analysis, high levels of TNF-α mRNA have also been found in the (PBMC) of such patients, reflecting a state of immunoactivation [7]. In spite of this activated state of the immunocompetent cells, an impaired TNF-α system can still be detected owing to binding of TNF-α protein to the abundant sTNF-R. As these receptors are metabolized and cleared by the kidney, one might

expect a normalization of the TNF-α/TNF-R balance after succesful kidney transplantation [8]. However, if factors other than clearance of the sTNF-R play a role, the imbalance of the TNF-α system would not improve after restoration of renal function [9]. In the present study we analyzed TNF-α protein, the sTNF-R 1 and -R 2, and TNF-α mRNA before and after kidney transplantation.

Patients, material and methods

Patients

In 16 patients (9 male, 7 famale; age 22–55 years, mean 32 years) all receiving a living-related kidney transplantation, we measured sTNF-R in peripheral blood and in urine before and during the first 6 days after transplantation. In PBMC TNF-α mRNA was measured. Before transplantation all but two patients were on renal replacement therapy (eight patients on haemodialysis, six patients on CAPD). Donations were from parent to child in eight cases and between siblings in eight cases (five identical, four haploidentical or better combinations). All grafts functioned immediately. No hyperacute, accelerated or acute rejections were observed. To evaluate the levels of sTNF-R over time, we measured serum and urine levels at 1 year after kidney transplantation in 20 other patients, with stable serum creatinine values, who used cyclosporine ($n = 10$) or azathioprine ($n = 10$) as maintenance immunosuppressive therapy. Healthy subjects ($n = 11$) were used as controls.

Sample preparation

Blood samples were collected in pyrogen-free tubes containing EDTA in a final concentration of 1 mg/ml from all patients, before and after transplantation daily until day 6. The samples were immediately centrifuged, plasma and cell fractions were separated and the plasma was stored at −80 °C. After transplantation urine samples were collected until day 6. Urine was centrifuged and the supernatant was stored at −80 °C until analysis. For the isolation of PBMC, the buffy coat was diluted in phosphate-buffered saline (PBS) solution and layered over a Ficoll-Isopaque gradient ($\delta = 1.077$). After centrifugation, the PBMC were removed from the interface, and washed twice with ice-cold PBS. Immediately following procurement, 2×10^6 cells were snap-frozen in liquid nitrogen and stored at −80 °C for RT-PCR analysis.

TNF-α protein and soluble TNF receptors

TNF-α in plasma was detected using four different commercially available ELISA kits (CLB, Amsterdam; Genzyme; Medgenix and Pharmingen). All kits detected TNF-α, but they differed in their detection limit. Especially at the lower levels the kits from Genzyme and Pharmingen were more sensitive. The detection limits were 10 pg/ml (CLB), 4 pg/ml (Genzyme), 12 pg/ml (Medgenix) and 4 pg/ml (Pharmingen). sTNF-R 1 and -R 2 in plasma and urine were measured using a double-sandwich ELISA (R & D Systems Europe, Abington, UK). The detection limit of this commercial kit was 15 pg/ml for sTNF-R 1 and -R 2. All ELISA techniques were performed following the manufacturer's instructions.

Isolation of mRNA and cDNA reaction

Messenger RNA from the TNF-α gene and keratin gene, a housekeeping gene, was isolated, reverse transcribed and subjected to PCR analysis. Messenger RNA extraction and transcription were performed as described previously [9]. Sequence-specific primers were used for amplification of the human TNF-α and keratin genes. Briefly, total RNA was extracted from PBMC by a modification of the guanidinium method. Total RNA was precipitated, pelleted and washed, followed by denaturation for 5 min at 80 °C and chilled on ice. First strand cDNA synthesis was performed from the isolated RNA with 0.5 μg hexanucleotides (Promega Corporation, Madison, Wis.) and transcribed with 1000 U Moloney murine leukemia virus reverse transcriptase (Gibco-BRL, Gaithersburg, Mo.) at 42 °C for 90 min in a total volume of 100 μl.

Competitive template RT-PCR

To estimate the initial relative amount of functional TNF-α mRNA in PBMC a competitive RT-PCR assay was used and comparison was made against the housekeeping keratin gene. The latter gene is assumed to be expressed at a constant level in PBMC. A 5-μl aliquot of cDNA sample and 5 μl of gene-specific competitive templates were added to 90 μl PCR mixture containing 10 m*M* Tris-HCl, pH 8.3, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* each of dATP, dCTP, dTTP, dGTP, 2 U Taq DNA polymerase (Promega) and 50 pmol of 5'- and 3'-sequence-specific primers.

To obtain a standard curve for TNF-α and keratin, known amounts of internal control fragment were added at different dilutions to constant amounts of sample cDNA for competitive coamplification with specific primers. The internal control was designed to generate a PCR product of a different size to allow differentiation between the amplified target and internal standard. Dilutions of the competitor template, ranging from 5 ag to 50 pg were coamplified with constant amounts of sample cDNA. Each reaction mixture was overlaid with 75 μl mineral oil (Sigma, St. Louis, Mo.) prior to PCR reaction in a DNA thermal cycler (Perkin Elmer 480, Branchburg, N.J.) under the following conditions. After a 5 min 94 °C denaturation step, samples were subjected to 40 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 2 min, and extension at 72 °C for 3 min. The last cycle was extended with 7 min at 72 °C. Positive control samples were produced by stimulating 10^6 human spleen cells with 1% phytohaemagglutinin (PHA)-M (Difco, Detroit, Mich.) for 4 h at 37 °C. Negative controls consisted of omission of reverse transcriptase from the cDNA synthesis reaction for each sample followed by amplification in PCR with the TNF-α and keratin primers, and the use of diethylpyrocarbonate-treated H_2O as a no-template reaction.

Following PCR, 16 μl PCR product was analysed by gel electrophoresis and the amount of products in relation to the internal control, and targets were determined for each individual reaction. The relative ethidium bromide intensity on the gel was measured by luminescence with a DC-40 camera in combination with analysis software (Kodak, Rochester, N.Y.). The logarithm of the ratio target/internal control was plotted as a function of the logarithm of the internal molar amount of the standard, and at ratio 1, the starting concentration of TNF-α and keratin cDNA prior to PCR was assumed to the equal to the known starting concentration of the competing internal control. The relative concentrations of TNF-α transcripts were divided by the relative concentrations of keratin. This represents the amount of TNF-α mRNA transcripts corrected for the amount of mRNA used for reverse transcription and the efficiency of each reaction.

Table 1 sTNF receptors R1 and R2 and mRNA TNF-α before and after kidney transplantation. Values are means ± SD (*n. d.* not done)

	sTNF-R1 (ng/ml)		sTNF-R2 (ng/ml)		mRNA (fg)
	Plasma	Urine	Plasma	Urine	
Controls	0.7 ± 0.1	n.d.	1.6 ± 0.5	n.d.	18
Pretransplantation	14.5 ± 6.5*	n.d.	15.4 ± 5.7*	n.d.	12
Post-transplantation					
Day 1	3.8 ± 1.4**	10.9 ± 5.6	6.5 ± 2.5**	7.2 ± 4.9	10
Day 2	3.2 ± 1.2	12.4 ± 4.2	5.2 ± 2.3	7.8 ± 3.6	6
Day 3	3.0 ± 1.1	9.1 ± 3.5	4.9 ± 2.0	7.2 ± 3.7	66
Day 6	3.0 ± 1.4	6.7 ± 2.9	4.8 ± 1.8	5.6 ± 0.9	9
At 1 year after transplantation					
Cyclosporin group	3.2 ± 1.3	1.8 ± 0.7	5.8 ± 1.8	5.8 ± 1.1	n.d.
Azathioprin group	2.5 ± 0.7	2.7 ± 1.1	4.0 ± 0.8	9.3 ± 1.8	n.d.

* $p < 0.001$ versus controls and versus day 1, ANOVA;
** $p < 0.001$ versus pretransplantation

Results

Serum creatinine and creatinine clearance

Mean serum creatinine was 804 μmol/l before operation (range 433–1334 μmol/l) and had decreased to 135 μmol/l (range 60–413 μmol/l, median 110 μmol/l) by day 6. The mean creatinine clearance was 60 ml/min on day 6 (range 20–117 ml/min).

At 12 months after kidney transplantation, patients using cyclosporine had stable renal function with a mean serum creatinine of 171 μmol/l (range 76–265 μmol/l). The creatinine clearance was 59 ml/min. Patients using azathioprine had a mean serum creatinine of 122 μmol/l (range 81–214 μmol/l) with a creatinine clearance of 84 ml/min.

TNF-α protein

Using the four different sandwich ELISA techniques we were able to detect TNF-α in the plasma of all patients. However, levels were only just above the detection limits of the kits and no significantly higher levels were found in any of the patients.

Soluble TNF receptors in serum and urine

The concentrations of sTNF-R1 and sTNF-R2 were high in the plasma from all patients before transplantation (sTNF-R1 14.5 ± 6.5 ng/ml, sTNF-R2 15.4 ± 5.7 ng/ml; Table 1. After transplantation sTNF-R1 levels decreased to 3.8 ± 1.4 ng/ml ($p < 0.001$) on day 1 and to 3.0 ± 1.4 ng/ml ($p < 0.001$) on day 6 (Table 1). In spite of the restoration of renal function, sTNF-R1 levels remained significantly higher than those of healthy controls (0.7 ± 0.1 ng/ml; $p < 0.001$). sTNF-R2 levels also showed a significant decrease after restoration of renal function: 15.4 ± 5.7 ng/ml ($p < 0.001$) on day 1 to 4.8 ± 1.8 ng/ml ($p < 0.001$) on day 6 (Table 1). These levels were also significantly higher than those of healthy controls (1.6 < 0.5 ng/ml; $p < 0.001$).

Patients on cyclosporine 1 year after kidney transplantation had sTNF-R1 levels (3.2 ± 1.3 ng/ml) not significantly different from those in patients on azathioprine (2.5 ± 0.7 ng/ml; NS). sTNF-R2 levels were also not significantly different in cyclosporine patients than in patients on azathioprine (5.8 ± 1.8 ng/ml versus 4.0 ± 0.8 ng/ml; NS). However all receptor levels, both in patients on cyclosporine and in those on azathioprine, were significantly higher than the levels in healthy controls ($p < 0.001$).

In the urine samples of patients shortly after transplantation sTNF-R1 levels were 10.9 ± 2.5 ng/ml on day 1 and 6.7 ± 2.9 ng/ml on day 6 (NS). sTNF-R2 levels in urine were 7.2 ± 4.9 ng/ml on day 1 and 5.6 ± 0.9 ng/ml on day 6 (NS). After 1 year, sTNF-R1 levels in urine were 1.8 ± 0.7 ng/ml (cyclosporine group) and 2.7 ± 1.1 ng/ml (azathioprine group; NS), while sTNF-R2 levels were 5.8 ± 1.1 ng/ml (cyclosporine) and 9.3 ± 1.8 ng/ml (azathioprine; NS).

We evaluated the urinary excretion of sTNF-R by calculating the fractional clearance of the receptors in relation to that of creatinine. Six days after renal transplantation the fractional clearances of sTNF-R1 was 9% and of sTNF-R2 5%. The fractional clearances of the sTNF-R decreased to 2% for both receptors at 1 year after transplantation. After 1 year there was no significant difference in fractional clearances for both receptors between the patients on cyclosporine and those on azathioprine (1.9 ± 0.1 versus 2.3 ± 0.1; NS).

TNF-α mRNA

In the PBMC of the patients receiving a living-related kidney we measured TNF-α mRNA using an RT-PCR method daily from the day before transplantation (day −1) through day 6 after transplantation. Levels of TNF-α mRNA are expressed as the TNF-α/keratin ratio

in order to compensate for the total amount of mRNA produced by the cells and the methods of isolation and amplification. By using this method we were able to detect TNF-α mRNA in both patients and healthy controls. Levels of TNF-α mRNA as well as the TNF-α/keratin ratio were not different in patients compared with healthy controls TNF-α mRNA 11 fg vs 18 fg; TNF-α/keratin ratio 284 vs 528; NS). The levels of TNF-α and the TNF-α/Keratin ratio did not change in the days following successful transplantation.

Discussion

After living-related kidney transplantation renal function is normalized in a very short period of time. Creatinine clearance increases from < 10 ml/min to 60–90 ml/min. In the months after transplantation graft function may be compromised by nephrotoxic drugs such as cyclosporin, low levels of rejection or urinary tract infections. In spite of this, renal function is stable in many patients. One year after kidney transplantation, we found a creatinine clearance of 59 ml/min in the cyclosporine group compared with 84 ml/min in the azathioprine group. In newly transplanted patients, restoration of renal function was accompanied by a rapid fall in plasma sTNF-R levels. However, the serum sTNF-Rlevels remained elevated in transplant recipients compared with healthy controls. In contrast plasma levels of TNF-α, measured by ELISA techniques, were comparable to those in healthy controls. The TNF-α mRNA expression of the PBMC was also comparable to that in the controls.

We found no consistent decrease in TNF-α mRNA after successful transplantation. However, there was still an imbalance in the TNF-α system, caused by elevated levels of sTNF-R with normal levels of TNF-α (protein and mRNA). TNF-α levels may be normal because of impaired production by PBMC or a discrepancy between serum levels and locally produced TNF-α. Locally produced TNF-α binds to its membrane-bound receptor resulting in production of the split products, the sTNF-R. This sheedding of TNF receptors is partly responsible for the increased serum levels and is an indication of the activation of the TNF-α system.

As the fractional clearance of the sTNF-R was only 5 to 9%, the rapid fall in plasma levels of these receptors after transplantation cannot be explained by increased renal function. Nevertheless, after successful renal transplantation the imbalance in the TNF-α system in patients with renal failure is improved. However, compared with healthy controls the persisting imbalance suggests an ongoing immunocompromised state of these patients after transplantation.

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