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Soluble tumor necrosis factor-receptors are not a useful marker of acute allograft rejection: a study in patients with renal or cardiac allografts

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Abstract In this study, we investigated soluble tumor necrosis factor receptor (sTNF-R) levels in plasma of patients with either a kidney or cardiac allograft when clinical suspicion of acute rejection was raised. In plasma of patients with acute renal graft rejection, the sTNF-R levels were strongly enhanced (20–150 ng/ml) as compared to plasma of patients with stable renal function. Following successful treatment of the rejection, a gradual decline in sTNF-R levels occurred with improving renal function, and an inverse correlation between creatinine clearance and sTNF-R was found. To determine whether the increase was caused by an accumulation of constitutively released sTNF-R and lack of clearance by the kidney, or whether the immunological process of the rejection caused the enhancement, we measured sTNF-R in patients suffering from acute cardiac graft rejection but with predominantly stable kidney function. Rejection of a cardiac graft did not lead to a significant enhancement of sTNF-R levels. However, treatment with ATG or OKT3 did cause enhanced sTNF-R levels, followed by a decline that reached starting values after 7 days. These results provide evidence that the immune reaction that occurs during rejection of a graft does not per se induce discernible changes in sTNF-R levels, whereas that induced by ATG or OKT3 does. Thus, sTNF-R levels are not a reliable marker in transplant recipient monitoring.

Key words Acute rejection, TNF receptors · TNF receptors, acute rejection · Heart transplantation, acute rejection, TNF receptors · Kidney transplantation, acute rejection, TNF receptors

Introduction

Two tumor necrosis factor (TNF)-binding proteins detected in urine and in serum [13, 25] have been identified by molecular cloning and crossreactive antibodies as being the soluble (s) forms of the receptors for TNF, and they are TNF-R55 and TNF-R75 [28]. Appropriate activation of immunoreactive cells, such as neutrophils and monocytes, leads to the release of sTNF-R, as shown in in vitro experiments [11, 19, 20]. In vivo, ele-

vated plasma sTNF-R levels are found in pathological situations, such as sepsis, experimental inflammation, and rheumatoid arthritis, as well as after TNF infusion in cancer patients [14, 15, 17, 26, 31]. Furthermore, strongly enhanced sTNF-R levels are found in patients with chronic renal failure [6] and in hemodialysis patients [18, 21], supporting the hypothesis that the kidney is involved in the clearance of sTNF-R from blood. The biological function of sTNF-R in plasma is not yet clear. It is conceivable that sTNF-R play a role in scavenging circulating TNF, resulting in a decreased inflammatory reaction [31].

In patients with acute allograft rejection, a cascade of immunological reactions occurs, among which is the local production of TNF [22, 23, 30]. TNF is thought to play an important role in the pathophysiology of allograft rejection because of its induction of procoagulant activity and its multiple effects as an inflammatory cytokine, resulting in amplification of the immune reaction [30]. One might expect that this reaction would lead to the release of sTNF-R. In this prospective clinical study, we investigated whether rejection of an allograft results in enhancement of sTNF-R in plasma. To this end, sTNF-R were measured in renal allograft patients with acute rejection. Since, in these patients, enhanced sTNF-R levels might coincide with malfunctioning of the kidney, we also investigated sTNF-R in cardiac allograft patients suffering from acute rejection.

Materials and methods

Patients

A group of 33 patients (16 women, mean age 39.6 ± 5.2 years; 17 men, mean age 46.4 ± 4.3 years) with suspected renal rejection were studied. Thirteen patients were excluded from further evaluation because of renal failure due to reasons other than acute graft rejection [acute tubular necrosis ($n = 1$), chronic rejection ($n = 2$), and rejection not ascertained by biopsy ($n = 3$)], or because of insufficient (< 5) number of samples ($n = 7$). The remaining 20 patients suffered from acute graft rejection as evidenced by biopsy. The patients received cyclosporin and prednisolone as basic immunosuppression. Episodes of rejection were treated either by ATG (rabbit derived, RIVM, Bilthoven, The Netherlands) or by methylprednisolone (MP). Rejection was diagnosed on the basis of clinical symptoms and confirmed by core needle biopsy. The 1st day of treatment for rejection was designated as $t = 0$. A group of 26 renal transplantat recipients with stable renal function served as controls.

A group of 18 patients with a cardiac allograft were included in this study (2 women, 27 and 55 years old; 16 men, mean age 47.9 ± 2.7 years). The patients received cyclosporin and prednisolone as basic immunosuppression. After cardiac graft transplantation, endomyocardial biopsies were taken regularly (according to the local protocol) and analyzed according to criteria of the International Society of Heart Transplantation [5]. Rejection grade 3A was followed by treatment with either ATG, OKT3, or MP.

To provide control data, blood samples from normal healthy individuals ($n = 100$) were taken.

Collection of samples

Blood samples from renal transplant recipients were taken in EDTA tubes at the first signs of rejection and daily during the antirejection therapy for at least 2 weeks. Sampling from heart transplant recipients was done three times a week during the first 3 weeks after transplantation, simultaneously with the myocardial biopsy, and daily during rejection treatment. Plasma was prepared by centrifugation at 500 *g* for 10 min at 4 °C. Samples were stored at −70 °C until use. To provide control data, blood samples from renal transplant recipients with stable renal function and from normal, healthy individuals were similarly processed.

sTNF-R Measurement

sTNF-R levels in plasma of patients were measured in an enzyme-linked immunological biological assay (ELIBA), as described previously [21]. Briefly, monoclonal antibody (mAb) htr20 (anti-TNF-R55) or utr4 (anti-TNF-R75) was coated (2 µg/ml) on immunoassay microtiter plates (Nunc, Roskilde, Denmark), followed by saturation with 1 % BSA. Subsequently, plasma samples (diluted 1:5) and peroxidase-labeled TNF (100 ng/ml for detection of sTNF-R55 and 500 ng/ml for detection of sTNF-R75) were added in duplicate wells. After an overnight incubation, the plates were washed and peroxidase activity was determined by addition of substrate. mAb htr20 and utr4, labeled TNF, and standards sTNF-R55 and sTNF-R75 were kindly provided by Drs. H. Gallati and M. Brockhaus (Hoffman-La Roche, Basel, Switzerland). The intra- and interassay variations were less than 10 %. The sensitivity of the assay was 400 pg/ml. The presence of less than 10 ng/ml TNF or lymphotoxin did not interfere with the detection of sTNF-R.

Statistical analysis

Data are given as mean ± SEM, unless stated otherwise. The Mann-Whitney U-test and the Wilcoxon matched-pairs signed rank test were used for the statistical analysis of data.

Results

sTNF-R Levels in controls and in graft recipients with stable organ function

Mean levels of sTNF-R55 and sTNF-R75 in healthy controls ($n = 100$) were below the detection limit (< 2 ng/ml). In plasma from renal graft patients with stable kidney function ($n = 26$), sTNF-R levels ranged from less than 2 to 11 ng/ml for sTNF-R55 and from less than 2 to 8 ng/ml for sTNF-R75. The mean levels of both sTNF-R55 (5.2 ± 0.5 ng/ml) and sTNF-R75 (3.1 ± 0.2 ng/ml) were higher than the levels found in normal healthy controls for sTNF-R55 and sTNF-R75.

Also, in plasma from cardiac allograft recipients without signs of acute rejection ($n = 18$), sTNF-R levels were enhanced ($P < 0.01$) as compared to those in healthy controls, ranging from 2.2 to 8.5 ng/ml for sTNF-R55 and from less than 2 to 4.2 ng/ml for sTNF-R75. In

Table 1 Summarized data of sTNF-R levels and of creatinine clearance in patients with renal failure

Patients	*n*	sTNF-R55		sTNF-R75		Clearance[a] Mean ± SEM
		Mean ± SEM	Range	Mean ± SEM	Range	
Total[b]	33	53.5 ± 7.4	11–140	23.1 ± 2.3	4–56	–
Excluded	13	51.1 ± 10.7	10–80	19.9 ± 4.2	4–28	–
Acute rejection	20	56.2 ± 8.4	11–140	25.2 ± 3.9	6–56	9.4 ± 2
After treatment[c]	20	18 ± 3	6–65	9.3 ± 1.6	2–16	43.8 ± 3.6

[a] Creatinine clearance determined in patients (ml/min)
[b] Peak plasma sTNF-R values (0–3 days after admission to hospital)
[c] sTNF-R values 14 days after acute rejection was diagnosed and subsequently treated

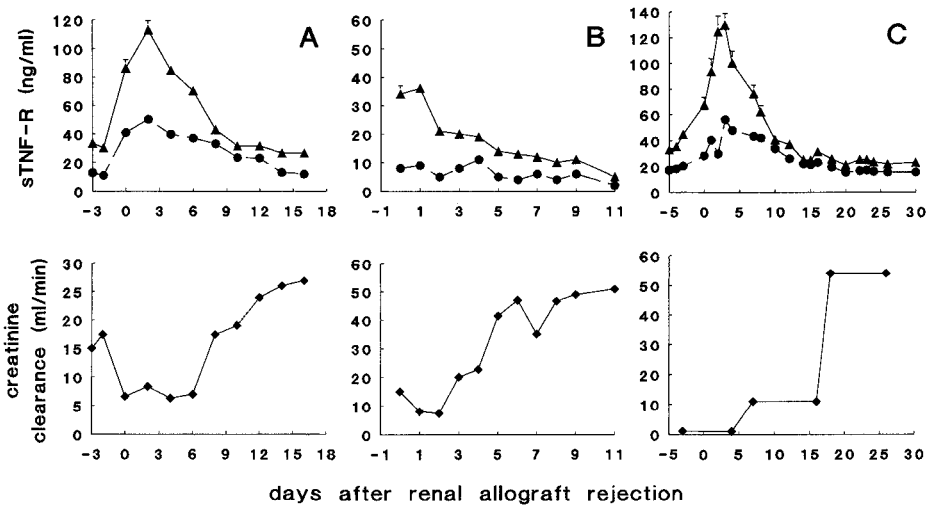

Fig. 1 Representative examples of serial sTNF-R levels (*upper panels*) and creatinine clearance (*lower panels*) during acute renal allograft rejection in three patients (A, B, C). sTNF-R levels were measured by ELIBA (-▲-, sTNF-R55, —●— sTNF-R75). The data represent the mean ± SD of triplicate samples. SD bars are omitted if they fall within the symbol

the course of the monitoring, minor fluctuations in sTNF-R levels were observed (data not shown).

sTNF-R Levels during acute renal graft rejection

Measurement of sTNF-R in plasma of all patients with suspected renal rejection ($n = 33$) showed that these levels were strongly enhanced as compared to sTNF-R levels in plasma of transplanted patients with stable renal function (Table 1). The sTNF-R levels of the patients excluded from this study did not differ significantly from those in patients with acute rejection. In patients with a low creatinine clearance because of acute rejection, sTNF-R levels were high (Table 1). After treatment of the rejection, kidney function improved and there was a concomitant significant decrease in sTNF-R levels ($P < 0.005$; Table 1). Since the sTNF-R levels varied considerably from patient to patient, Fig. 1 shows the sTNF-R levels and creatinine clearance of three representative patients. The sTNF-R levels are shown to be inversely correlated with creatinine clearance.

Since therapy with ATG or with OKT3 induces the production of TNF [1, 7, 9] and since mAb anti-CD3 administration in mice induces an enhancement of sTNF-R [4], we decided to study the effects of ATG as compared to those of MP on sTNF-R levels. As shown in Fig. 2, ATG did not lead to an enhancement of sTNF-R as measured on the day following treatment. Furthermore, no significant differences were observed between sTNF-R levels in renal allograft recipients treated with ATG, MP, or a combination of ATG and MP, as measured either on the day following treatment or 14 days after treatment (Fig. 2).

sTNF-R Levels during acute cardiac allograft rejection

The above-mentioned data could not provide a conclusive answer as to whether or not rejection increases sTNF-R levels because malfunctioning of the kidney might interfere. Therefore, sTNF-R levels were measured in the plasma of 12 patients with a cardiac graft who had had at least one rejection episode but who had stable kidney function. A total of 26 rejection episodes were investigated. sTNF-R levels at the onset ($t = 0$) of rejection (sTNF-R55: 6.5 ± 0.5 ng/ml; sTNF-R75: 3.9 ± 0.4 ng/ml) were not significantly altered as compared with sTNF-R levels measured before (1–7 days)

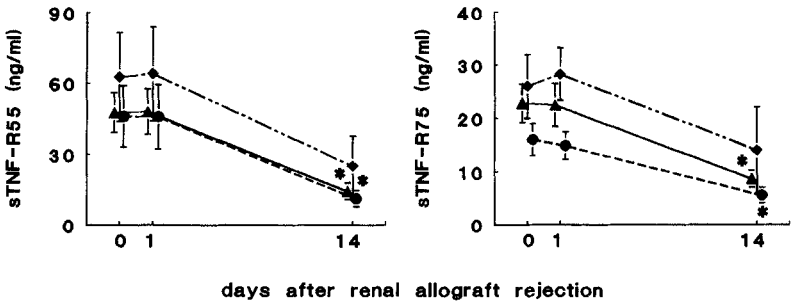

Fig. 2 sTNF-R levels in renal allograft recipients during treatment with ATG (-▲-), MP (—●—), or a combination of ATG and MP (-◆-). sTNF-R levels were measured in plasma at the onset of rejection (*0*), 1 day after rejection, and 14 days after rejection. At $t = 0$, treatment was started with ATG ($n = 8$, * $P < 0.012$ vs previous time point), with MP ($n = 8$, * $P < 0.012$ vs previous time point), or with a combination of ATG and MP ($n = 3$, not enough cases for statistical evaluation). sTNF-R levels in the groups of patients treated with ATG or with MP were not statistically different and there were not enough cases of combined ATG and MP for statistical evaluation

rejection (sTNF-R55: 6.5 ± 0.5 ng/ml; sTNF-R75: 3.6 ± 0.2 ng/ml). Furthermore, sTNF-R levels between rejection episodes were stable, irrespective of the minor fluctuations mentioned above.

Effect of ATG and OKT3 on sTNF-R levels in cardiac allograft recipients

We studied the effect of ATG or OKT3 as antirejection therapy in patients with acute cardiac allograft rejection. Both treatment with ATG and with OKT3 caused an enhancement of sTNF-R as measured on the day following treatment (Fig. 3). These elevations could not be ascribed to an impairment of renal function since daily measurement of serum creatinine levels showed no significant changes during the rejection and treatment period (Fig. 3). This enhancement was not observed when patients were treated with MP. After 7 days the levels were reduced to basal levels. In Fig. 4, the sTNF-R levels and serum creatinine of two patients, who repeatedly received ATG are shown. As can be seen, each ATG injection was followed by an elevation of sTNF-R, while serum creatinine remained constant.

Discussion

A growing number of studies have been published reporting enhanced sTNF-R levels during inflammatory reactions. In the present, prospective, clinical study we demonstrated that the immune reaction causing rejection of a renal or a cardiac allograft resulted in no detectable rise in sTNF-R levels in plasma. However, treatment with ATG or OKT3 induced a marked elevation in both sTNF-R55 and sTNF-R75. This enhancement was only observed in cardiac allograft recipients, not in renal allograft recipients. Patients with kidney malfunction showed strongly enhanced sTNF-R levels as compared to renal transplant recipients with stable kidney function. Therefore, changes in the kidney function most likely obscured relatively small fluctuations in sTNF-R levels in these patients. Apparently, treatment with ATG or OKT3 induced a more profound systemic inflammatory response than allograft rejection, something that was also reflected in the more severe clinical symptoms (e.g. fever, nausea) of these patients during treatment.

Peak levels of sTNF-R can be expected within the first few hours following the first therapy in response to cell activation [4]. Although we missed these peak levels since samples from the patients were taken daily, the enhancement was still present 1 day after the first treatment. These clinical data are in agreement with results obtained in previous studies using a mouse model in which administration of mAb anti-CD3 to mice caused an enhancement of sTNF-R [4]. In addition, in in vitro experiments, it was found that isolated peripheral blood cells constitutively released both sTNF-R. This release was enhanced upon activation by phorbol esters, IL-10, endotoxin, and also by mAb anti-CD3 [19, 20].

Basal sTNF-R levels in both cardiac and renal allograft recipients with stable organ function were significantly enhanced as compared to normal, healthy individuals. This enhancement is most likely a consequence of reduced renal function due to regular treatment with cyclosporin [24, 32].

While looking for new parameters to monitor allograft rejection, the kinetics of soluble receptors other than TNF-R, such as soluble interleukin 2 receptor [16, 29] and soluble ICAM-1 [27] have been analyzed in plasma during renal and cardiac allograft rejection. As with sTNF-R levels during rejection, enhanced levels could not be discerned for acute rejection or other complications since levels were mostly related to kidney graft function or since they were enhanced during both rejection and infection [8].

As mentioned earlier, enhanced sTNF-R levels have been found in a large number of pathological condi-

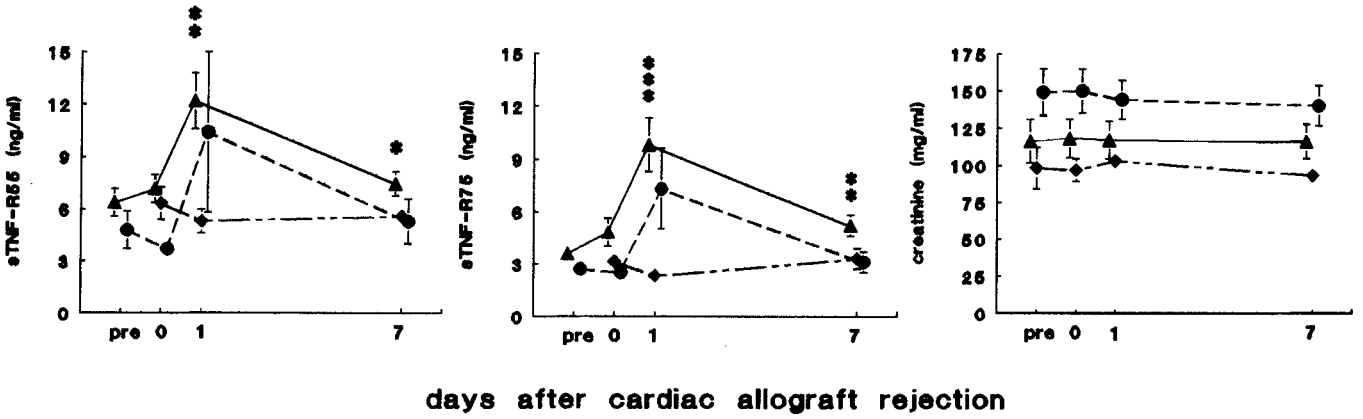

Fig. 3 sTNF-R levels are enhanced in cardiac allografts recipients after treatment with ATG (-▲-) OKT3 (-●-) but not with MP (-◆-), whereas creatinine remains constant. sTNF-R levels in plasma and serum creatinine were measured before (*pre*) cardiac rejection, at the onset of rejection (*0*), 1 day after rejection, and 7 days after rejection. At $t = 0$, treatment was started with ATG ($n = 10$, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs previous time point), OKT3 ($n = 3$, not enough cases for statistical evaluation), or MP ($n = 4$, not enough cases for statistical evaluation)

tions. This enhancement, however, is only minor compared to the strongly enhanced sTNF-R found in patients with impaired renal function. These sTNF-R may be derived from monocytes and endothelial cells that constitutively release sTNF-R [20], leading to an accumulation of sTNF-R in plasma. The strong inverse correlation between renal function and sTNF-R levels supports the hypothesis that the kidney is involved in the clearance of sTNF-R from blood. Further evidence of a major role of the kidney in the clearance of sTNF-R was given by Bemelmans et al., who found a prolonged presence of sTNF-R in bilateral, nephrectomized mice after lipopolysaccharide (LPS) challenge [3]. It is worth noting that in these mice, sTNF-R levels were higher than those in nephrectomized mice without LPS challenge, indicating that activation in such mice can lead to even more enhanced sTNF-R levels. The deterioration of renal function during rejection causes a decreased clearance of sTNF-R during the rejection that is compensated, although apparently less efficiently, by the liver, as shown in experiments performed in a mouse model [Bemelmans, personal communication].

It has been reported that serum TNF levels rise shortly before rejection, peak on the day of rejection, and return to basal levels within 2–3 days [30]. However, we failed to detect any biologically active TNF during rejection (data not shown). These results are in accordance with a previous study in which the majority of patients, having rejected their renal allograft, had TNF plasma levels below the detection limit of 5–10 pg/ml [9]. It is conceivable that this discrepancy is caused either by the use of different assays for TNF measurements [12] or by the different time points at which plasma is collected. In our TNF assay, in which biologically active TNF was measured, TNF is most likely bound to the sTNF-R and no longer detectable. Thus, sTNF-R might offer protection against systemic circulating TNF. However, the role of sTNF-R in vivo is still speculative. As suggested by Aderka et al. [2], complexes of TNF with sTNF-R could provide a slow release reservoir of TNF. This hypothesis was further supported by De Groote et al., who demonstrated in in vi-

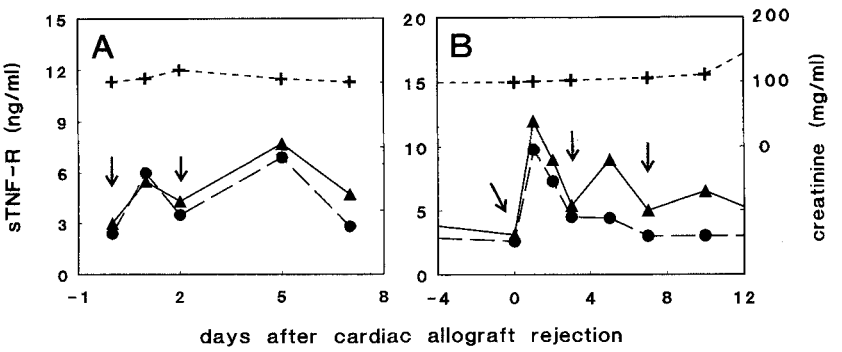

Fig. 4 Repeated treatment with ATG caused repeated enhancement of sTNF-R levels. *Arrows* indicate ATG treatment in two cardiac allograft recipients (A and B). sTNF-R levels were measured by ELIBA (-▲- sTNF-R55, -●- sTNF-R75). Serum creatinine was given in mg/ml (- + -)

tro experiments that the trimeric form of TNF, which is the biologically active one, is stabilized by sTNF-R [10]. The clinical importance of such a prolonged presence of TNF in the pathology of patients who have rejected a renal graft remains to be elucidated.

To summarize, we showed in this study that acute rejection of a cardiac allograft did not induce enhancement of sTNF-R. In these patients we did, however, observe an enhancement of sTNF-R in response to treatment with ATG or with mAb OKT3. In contrrast, sTNF-R in patients with renal graft rejection were so strongly enhanced due to impaired renal function that enhancement caused by rejection or by treatment with ATG or OKT3 could not be demonstrated. Taken together, these results indicate that the monitoring of sTNF-R levels in plasma is not useful in the diagnosis of acute allograft rejection.

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