The TNF-α system in heart failure and after heart transplantation

Plasma protein levels, mRNA expression, soluble receptors and plasma buffer capacity

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Background The two soluble tumour necrosis factor (TNF) receptors (sTNF-R1, sTNF-R2) can bind TNF-α, which is a cytokine with cardiodepressant properties. In heart failure and after heart transplantation, the TNF-α system is unbalanced, due to elevated levels of sTNF receptors.

Aim To assess the activity of the TNF-α system in patients with heart failure and after heart transplantation.

Methods We measured TNF-α mRNA expression of peripheral blood mononuclear cells, plasma levels of TNF-α and sTNF reverse transcriptase receptors, using polymerase chain reaction and ELISA and performed a TNF-α binding capacity analysis, quantitating the buffer capacity of patients' plasma.

Results In 11 patients with heart failure and in 15 cardiac allograft recipients, the TNF-α mRNA expression was comparable to controls. This level of mRNA was not accompanied by detectable TNF-α plasma levels. Significantly higher sTNF receptors levels were found in patients: ($P<0.001$; ANOVA). The TNF-α binding capacity of patients' plasma was significantly increased, which led to decreased TNF-α recovery ($P<0.05$). Both sTNF receptors showed a linear correlation with serum creatinine (sTNF-RI: $r=0.92$; sTNF-R2: $r=0.82$, $P<0.001$).

Conclusions The TNF-α mRNA expression and plasma levels show that the 'peripheral' TNF-α system is not activated. The high sTNF-receptors levels and their elevated TNF-α binding capacity, resulting in decreased TNF-α bioavailability, may contribute to an immunosuppressed state in these patients.
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Key Words: TNF-α, soluble TNF receptors, heart failure, heart transplantation.

Introduction

Tumour necrosis factor-α (TNF-α) is a pro-inflammatory cytokine with cardiodepressant properties[1]. In dilated cardiomyopathy, in end-stage ischaemic heart disease and after heart transplantation elevated plasma levels of TNF-α may be found[2–5]. In addition, we and others reported significant expression of TNF-α mRNA within the allograft after heart transplantation and we also showed that this level of TNF-α gene expression correlated with the efficacy of steroid anti-rejection therapy[6,7]. In general, activated peripheral blood mononuclear cells are the main source of TNF-α. In patients with heart failure, a positive correlation is found between the severity of heart failure and TNF-α levels[8]. Excessive catecholamine production and generalized endothelial dysfunction in these patients may contribute to the activation of TNF-α, both at the peripheral and central cardiac level[9]. In heart transplant patients, TNF-α can be produced not only by activated recipient-derived, graft-infiltrating cells, but also by donor cells, e.g. myocytes and endothelium[2,11–13]. In spite of local TNF-α production in the allograft, no clear relationship between sTNF receptors and rejection has been established[14]. All cells, except erythrocytes and resting T-cells, have

membrane-bound TNF receptors R1 and R2, which regulate the biological activity of TNF-*a*. After binding with the receptor, proteolytic cleavage results in split products, the soluble TNF receptors R1 and R2. High levels of sTNF receptors are found in patients after failed and successful heart transplantation, indicating that produced TNF-*a* is bound to its receptors, resulting in the production of these split products. The circulating sTNF receptors can bind 1–3 free TNF-*a* molecules, thereby acting as a buffer for locally produced TNF-*a*[15]. Under physiological circumstances free TNF-*a* is in balance with its sTNF receptors. However, under a variety of clinical conditions the TNF-*a*/sTNF receptor ratio may be disturbed[16]. Apart from heart failure, conditions in which the TNF-*a* system is out of balance are sepsis[17] and compromised renal function (renal failure, haemodialysis)[18]. In sepsis, TNF-*a* production overcomes the buffer capacity of the sTNF receptors and free TNF-*a* can be detected in the plasma. In renal failure, the impaired clearance of the sTNF receptors is the reason for the unbalanced TNF system, while during haemodialysis both TNF-*a* production by activated peripheral blood mononuclear cells and impaired clearance of the receptors, disturbs the balance between TNF-*a* and its receptors. Moreover, generalized arteriosclerosis and nephrotoxic immunosuppressive drugs cause impaired renal function and lead to decreased clearance of the sTNF receptors in both heart failure and cardiac allograft recipients. This results in a disturbed TNF-*a*/sTNF receptor ratio.

In the present study, we evaluated the nett balance of the factors: production and clearance. Therefore, we analysed the potential TNF-*a* production by measuring its mRNA expression in peripheral blood mononuclear cells, free plasma protein levels and sTNF receptors R1 and R2. In order to evaluate the bioactivity of the sTNF receptors, we measured the plasma binding capacity for recombinant TNF-*a* in patients' and control plasma, after incubation with free, active, recombinant human TNF-*a*.

Patients and Methods

In 11 patients with heart failure (eight males and three females, mean age 66 years, range 22–76, mean serum creatinine 107 μmol . l^{-1}, range 53–390), we measured TNF-*a* mRNA expression of peripheral blood mononuclear cells, free plasma TNF-x and soluble TNF receptors (sTNF-R1 and sTNF-R2). The patients suffered from heart failure, due to ischaemic heart disease (multiple myocardial infarctions, n=6), dilated cardiomyopathy after alcohol abuse (n=2), mitral valve stenosis (n=2) and congenital heart disease (n=1). They had been admitted to hospital because of progression of heart failure. They were classified according to the New York Heart Association Class III (n=9) or IV (n=2). Four patients underwent heart catheterization. During this procedure the left ventricular ejection fraction, and

right and left sided pressures were measured (mean pulmonary arterial pressures ranged from 21–45 mmHg). In seven patients, the left ventricular ejection fraction was determined by radionucleotide angiography. The mean left ventricular ejection fraction was 18% (range: 15–20%) in nine patients. The two patients with mitral valve stenosis, however, had a left ventricular ejection fraction of 65–70%. Medication consisted of digoxin (n=5), nitrates (n=5), calcium entry blockers (n=5), beta-blockers (n=2), diuretics (n=6) and ACE inhibitors (n=9). Two patients used amiodarone.

TNF-*a* measurements were also performed in 15 cardiac allograft recipients (11 males, four females; mean age 56 years, range 21–67) with stable renal function (mean serum creatinine: 113 μmol . l^{-1}; range 63–140 μmol . l^{-1}, creatinine clearance: 90 ± 27 ml . min^{-1}). Echocardiography at the time of blood sampling revealed normal systolic left ventricular function. There were no signs of rejection or infection. Time after transplantation ranged from 4–127 months (median 59 months). All heart transplant patients received cyclosporine and steroids as maintenance immunosuppressive therapy. The mean whole blood trough cyclosporine level was 158 ng . ml^{-1} (range 85–320 ng . ml^{-1}, median 145). Twelve healthy subjects served as controls (four males, mean age 30 years, range 24–52, creatinine <100 μmol . l^{-1}).

Sample preparation

Blood samples were collected in pyrogen-free tubes, containing EDTA in a final concentration of 1 mg . ml^{-1}. The samples were immediately centrifugated, plasma and cell fractions were separated and the plasma stored at − 80 °C until analysis. For isolation of peripheral blood mononuclear cells, the buffy coat was diluted in phosphate-buffered saline and layered on a Ficoll-Isopaque gradient. After centrifugation, the mononuclear cells were removed from the interface, and washed twice with ice-cold phosphate-buffered saline. Immediately after procurement, 2×10^6 cells were snap-frozen in liquid nitrogen and stored at − 80 °C for reverse transcriptase polymerase chain reaction.

Isolation of mRNA and copy-DNA reaction

Total RNA was extracted from snap-frozen samples by a modification of the guanidinium method (Chromczynski[19]), described by Baan[20]. Cells were homogenized in 500 μl 4 mol . l^{-1} guanidinium-isothiocyanate in the presence of 20 μg poly A (Boehringer, Mannheim, Germany). The solution was extracted once with phenol, phenol-chloroform-isoamylalcohol [25:24:1] and chloroform-isoamylalcohol [24:1], respectively. Total RNA was precipitated with 600 μl 2-propanol and 35 μl 3 mol . l^{-1} sodium acetate (pH 5·2) at − 20 °C for 18 h. Precipitates were pelleted

(a)

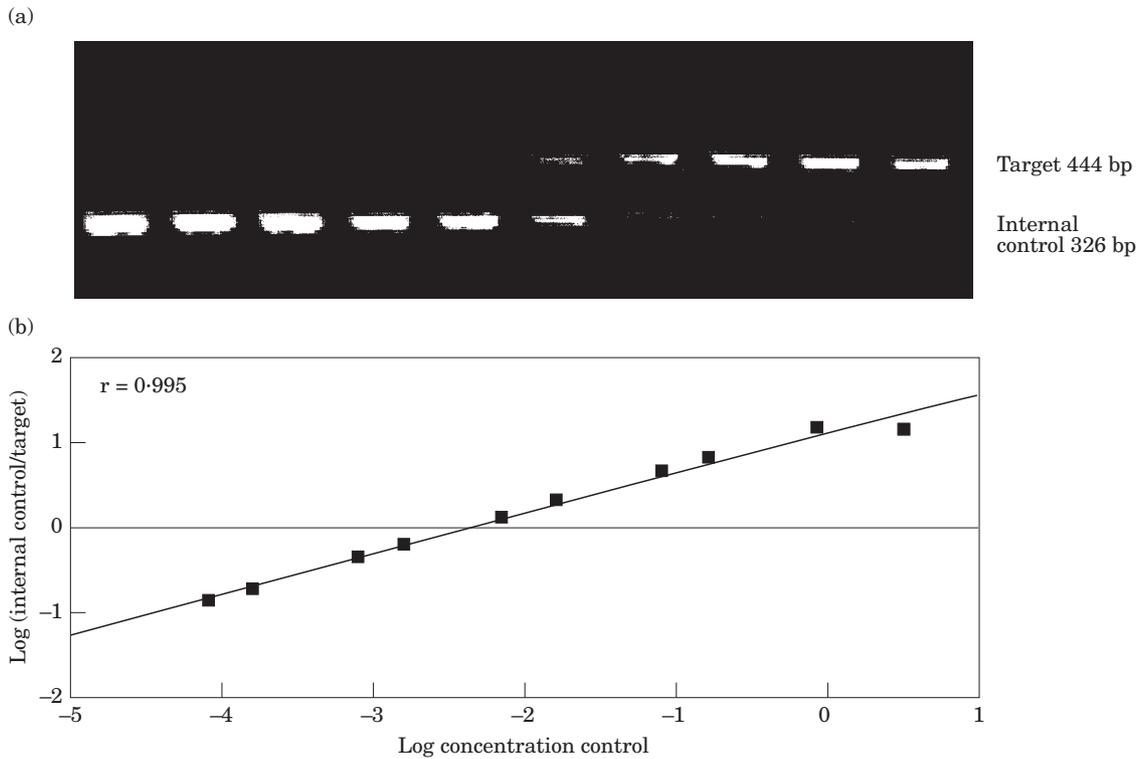

Target 444 bp

Internal
control 326 bp

(b)

Figure 1 **(a) Typical example of quantitative reverse transcriptase polymerase chain reaction analysis of TNF-*α*. After electrophoresis, the ethidium bromide stained agarose gel shows two polymerase chain reaction products of TNF-*α* per lane, representing the amplified target (444 bp) and internal control (326 bp). (b) The log of the amplified TNF-*α* to competitor TNF-*α* is graphed as a function of the log of the known amount of competitor added to polymerase chain reaction. At the point where target cDNA and internal control are in equivalence (ratio=1), the starting concentration of TNF-*α* cDNA prior to polymerase chain reaction is equal to the known starting concentration of the competing internal control.**

at $10\,000 \times g$ at 4 °C and washed once with 500 µl ice-cold 80% ethanol. Air-dried pellets were resuspended in 50 µl diethylpyrocarbonate treated-H_2O. Total RNA was denatured for 5 min at 80 °C and then chilled on ice. First stand cDNA synthesis was performed from the isolated RNA with 0·5 µg hexanucleotides (Promega Corporation, Madison, WI, U.S.A.) and transcribed with 1000 U Moloney murine leukemia virus reverse transcriptase (Gibco-BRL, Gaithersburg, MD, U.S.A.) at 42 °C for 90 min in a total volume of 100 µl. The reaction mixture consisted of 20 µl $5 \times$ MMLV-RT buffer (250 mmol . 1^{-1} Tris-HCl pH 8·3, 15 mM $MgCl_2$, 375 mM KCI), 5 µl (10 mM) dNTP, 400 U of RNAsin (Promega) and 10 µl 0·1M DTT.

Competitive template reverse transcriptase polymerase chain reaction

Sequence-specific primers were used for amplification of the human TNF-*α* gene (sense primer: 5′ GAG TGA CAA GCC TGT AGC CCA TGT TGT AGC A 3′ and anti-sense primer: 5′ GCA ATG ATC CCA AAG TAG

ACC TGC CCA GAC T 3′). Polymerase chain reaction primers detecting transcripts for the human housekeeping gene, keratin, were used as an internal control to monitor mRNA extraction and cDNA amplification[22]. To estimate the relative initial amount of functional TNF-*α* mRNA in peripheral blood mononuclear cells a competitive template reverse transcriptase polymerase chain reaction 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 haemato-poetic cells[21]. Five µl cDNA samples and 5 µl of gene-specific competitive templates were added to 90 µl polymerase chain reaction mixture containing 10 mM Tris-HCl pH 8·3, 1·5 mM $MgCl_2$, 0·2 mM 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 the TNF-*α* and keratin, known amounts of internal control fragment were added in different dilutions to constant amounts of sample cDNA for competitive co-amplification with specific primers (Fig. 1(a)). The internal control was designed to generate a polymerase chain reaction product of a different size to allow differentiation
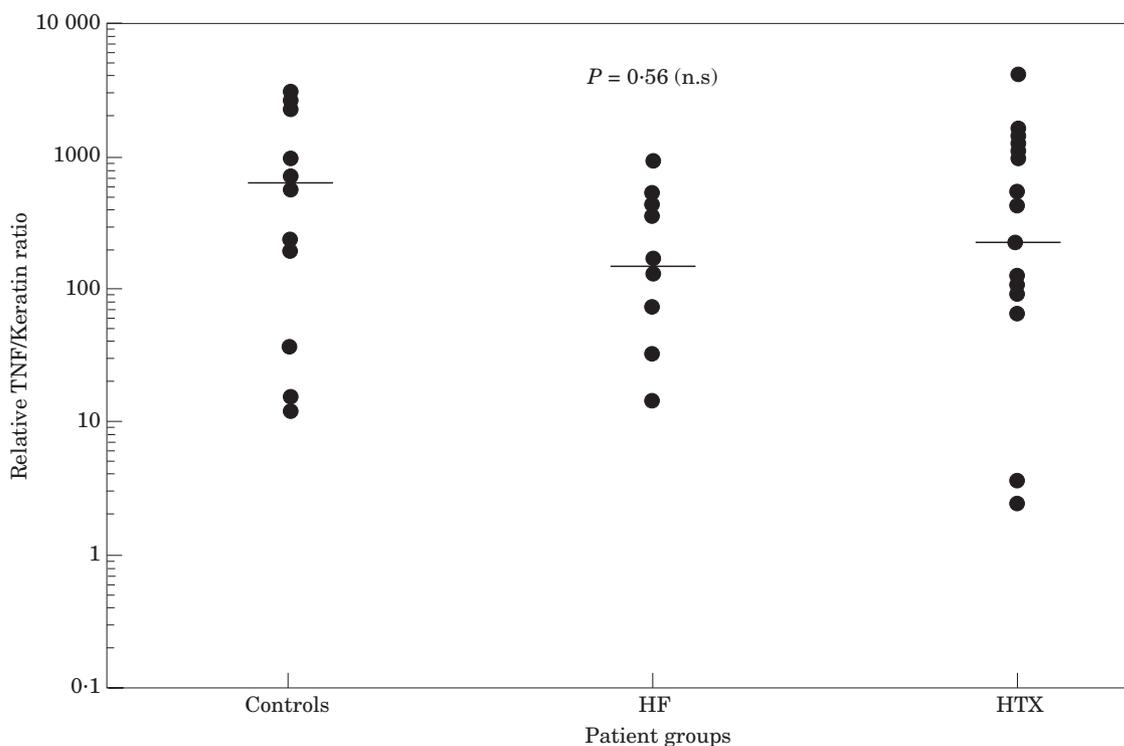
Figure 2   **TNF-α mRNA production by peripheral blood mononuclear cells obtained from patients with heart failure (HF), after heart transplantation (HTX), compared to healthy controls, denoted as the relative TNF-α/Keratin ratio, measured by competitive template reverse transcriptase polymerase chain reaction.**

between the amplified target and internal standard (TNF-*a*: 444 and 326 bp) and keratin (target=218 bp, internal control=160 bp). Each reaction mixture was overlaid with 75 μl mineral oil (Sigma, St. Louis, MO, U.S.A.) prior to polymerase chain reaction in a DNA thermal cycler (Perkin Elmer-480, Branchburg, NJ, U.S.A.) 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 for 7 min at 72 °C. Positive control samples were produced by stimulating 10^6 human spleen cells with 1% phytohemagglutinin (PHA)-M (Difco, Detroit, Ml, U.S.A.) 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 polymerase chain reaction with the TNF-*a* and keratin primers, and the use of diethylpyrocarbonate treated-H_2O as no-template reaction. Following polymerase chain reaction, 16 μl of polymerase chain reaction product was analysed by gel electrophoresis and the amount of product by the internal control and targets were determined for each reaction. The relative ethidium bromide intensity on gel was measured by luminescence with a DC-40 camera in combination with analysis software (Kodak, Rochester, NY, U.S.A.). The logarithm of the ratio target/internal

control is graphed as a function of the logarithm of the internal molar amount of the standard and at ratio 1. The starting concentration of the TNF-*a* and keratin cDNA prior to polymerase chain reaction is assumed to be equal to the known starting concentration of the competing internal control (Fig. 1(b)). The relative concentration of TNF-*a* transcripts were divided by the relative concentration of keratin. This represents the amount of TNF-*a* mRNA transcripts corrected for the amount of mRNA used for reverse transcription and the efficacy of each reaction.

TNF-α and soluble receptors R1 and R2 by ELISA

TNF-*a* in plasma was measured by a commercially available enzyme linked immuno sorbent assay (ELISA) (Genzyme, Brussels, Belgium). The detection limit is 4 pg . ml^{-1}; the coefficient of variation is 5–10%. The plasma soluble TNF receptors R1 and R2 are detected in the same blood sample, using a double sandwich ELISA technique (R&D Systems Europe, Abingdon, U.K.). The detection limit for both receptors is 15 pg . ml^{-1}, the coefficient of variation is less than 5% (sTNF-R1) and less than 10% (sTNF-R2).
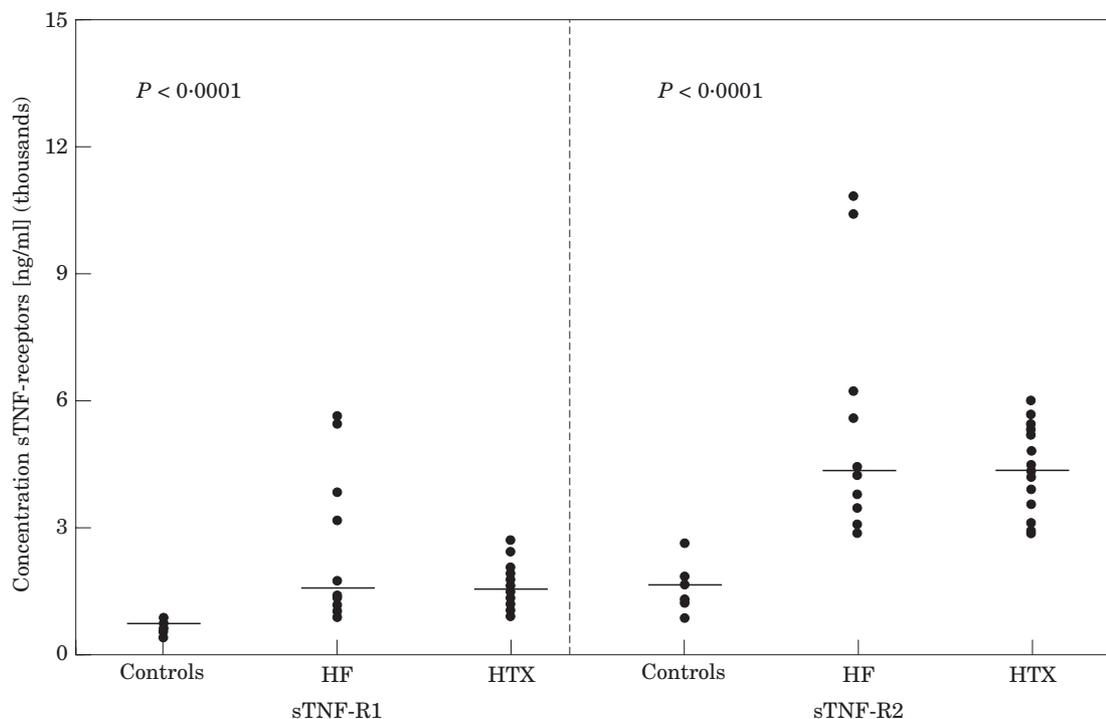
Figure 3 Plasma levels of soluble TNF-receptors R1 and R2 (ng . ml^{-1}) in patients with heart failure (HF) and after heart transplantation (HTX), compared to controls, measured by ELISA.

Plasma TNF-α binding capacity after incubation

Plasma sTNF receptors can bind 1–3 free TNF-α molecules. TNF-α binding assays can be performed using various bio-assays[15]. We performed the TNF-α binding assay, measuring the remaining TNF-α after incubation with patient or control plasma using a commercially available ELISA. A standard curve was constructed using different concentrations of the recombinant TNF-α (final concentration 300 pg . ml^{-1}). The plasma TNF-α binding capacity can be expressed as TNF-α recovery. TNF-α recovery is the residual TNF-α concentration (after incubation of recombinant TNF-α with sTNF receptor containing plasma) divided by the initial concentration of TNF-α × 100%. After incubation of 0·5 ml plasma with recombinant human TNF-α at room temperature, the ELISA test was performed following the manufacturer's instructions. Calculation of the residual TNF-α concentration was completed by extrapolation to the standard curve.

Statistics

The results are denoted as mean ± standard deviation, or median with range, whenever appropriate. The Mann–Whitney U test was used for non-parametric analysis, *P*-values <0·05 were considered significant. The data of the soluble TNF-receptors were compared using the ANOVA. The TNF-α recovery is evaluated by the Dunn's multiple comparison test.

Results

TNF-α mRNA expression was detectable in all blood samples, including those obtained from healthy controls. Therefore, we performed a quantitative competitive template reverse transcriptase polymerase chain reaction analysis to determine the level of TNF-α mRNA expression. Using the reverse transcriptase polymerase chain reaction, we found no statistical difference among groups in expression of the positive control housekeeping gene, keratin. This indicates that the integrity of the mRNA in the analysed patient groups and in controls was the same (*P*>0·05, Kruskal–Wallis). Relative amounts of initial TNF-α mRNA were individually normalized to the corresponding keratin levels which permitted more accurate comparison of TNF-α gene transcript levels. Relative TNF-α mRNA levels in peripheral blood mononuclear cells were not different in patients with heart failure or after heart transplantation compared to controls (mean TNF-α/keratin ratio: 129 (heart failure) vs 186 (heart transplantation) *P*=0·57; 129 (heart failure) vs 598 (controls), *P*=0·56; 186 (heart transplantation) vs 598 (controls), *P*=0·45; Mann–Whitney U, Fig. 2). This level of the TNF-α mRNA-expression did not result in detectable TNF-α levels in plasma in either the patient groups or the healthy
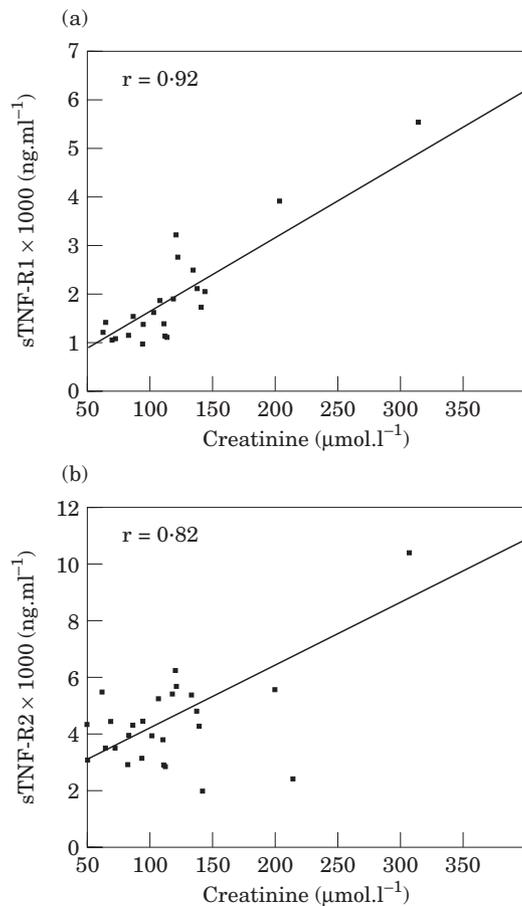
(a)

(b)

Figure 4 (a) and (b). **Correlation between serum creatinine (μmol . l⁻¹) and soluble TNF-receptor R1 (ng . ml⁻¹) (a) and R2 (ng . ml⁻¹) (b) in patients with heart failure and after heart translanation.**

controls (all samples: TNF-a <4·0 pg . ml⁻¹, which is the lowest detection limit of the ELISA). However, significantly higher levels of the sTNF receptor R1 and R2 were found in plasma obtained from patients with heart failure after heart transplantation compared to control plasma. In patients with heart failure, the mean plasma level of sTNF-R1 was 2·6 ± 1·7 and of sTNF-R2: 5·6 ± 2·7 ng . ml⁻¹. In plasma obtained from heart transplant recipients, the mean level of sTNF-R1 was 2·9 ± 1·3 and of sTNF-R2: 4·9 ± 2·1. In controls, the mean plasma level of sTNF-R1 is 0·7 ± 0·1 ng . ml⁻¹, and of sTNF-R2: 1·6 ± 0·5 ng . ml⁻¹, P<0·0001; ANOVA (Fig. 3). Both sTNF receptors showed a linear correlation with serum creatinine levels (sTNF-R1: r=0·92, sTNF-R2: r=0·82, P<0·001, Fig. 4(a) and (b)). Our results suggest that the absence of free, detectable plasma TNF-a is the consequence of the surplus of circulating sTNF receptors. We therefore speculate that produced TNF-a is bound to the surplus of circulating sTNF receptors. To evaluate the biological activity of this surplus of sTNF receptors we performed a TNF-a recovery test. A fixed amount of recombinant TNF-a was incubated with both patient plasma (heart failure

and after heart transplantation) and control plasma. TNF-a recovery was significantly reduced after incubation with patient plasma compared to incubation with plasma obtained from healthy controls (79 ± 16% (heart failure), 75 ± 5% (after heart transplantation) vs 100 ± 7%; P<0·05; ANOVA). Fig. 5.

Discussion

The TNF-a system has been reported to be out of balance, both in heart failure and renal insufficiency, and after organ transplantation[4,5,8,9,14,18,22]. Activation of the system may result in further progression of heart failure[1–3], while impairment of the TNF-a system may be the cause of higher susceptibility for infections and the development of malignancies. In an attempt to assess the biological significance of the disturbed TNF-a system, we studied various components of the TNF-a system in patients with heart failure and after heart transplantation. We found an unbalanced TNF/sTNF receptor ratio in both patient groups. This disturbance was mainly caused by high levels of biologically active soluble TNF receptors. These sTNF receptors are split products of the TNF membrane receptor, which are shed after TNF-a binding. Virtually all cells carry TNF membrane receptors. Cell-activation or TNF binding results in increased expression of the TNF receptors[23,24]. Binding to the membrane-bound TNF receptor R1 or R2 results in a cascade of events. Depending on interaction with R1 or R2, TNF-a binding leads to apoptosis, tumour cell lysis, haemorrhage necrosis, T-cell proliferation or insulin resistance[24].

High amounts of TNF-a have been found in plasma of patients with chronic heart failure, depending on the severity of heart failure[5]. However, a wide range between individuals can be found, known as genetically low or high TNF-a producers[25].

The lower detection limit of 4 pg . ml⁻¹ of our ELISA kit led us to anticipate finding TNF-a in the plasma from patients with heart failure. However, we detected no free plasma TNF-a. Commercially available ELISA kits show a wide range of measurement results, which make comparison between two studies hardly possible[15]. Besides that, the high levels of sTNF receptors were able to bind 25–30% of free, active TNF-a in-vitro, and thus could prevent measurement of free TNF-a. In previous studies we described the TNF-a system in end-stage renal failure and after kidney transplantation[18,22]. In patients on chronic haemodialysis, as well as patients on peritoneal dialysis and with end-stage renal failure, the TNF/sTNF balance was disturbed, due to high levels of mRNA-TNF-a expression (only in patients on haemodialysis) and high levels of sTNF receptors. Uraemia itself is known to be a potent initiator of TNF-a production, but in chronic haemodialysis, the blood-membrane interaction is seen as the main TNF-a production inductor. Impaired renal clearance has been found to give a positive correlation with sTNF receptor
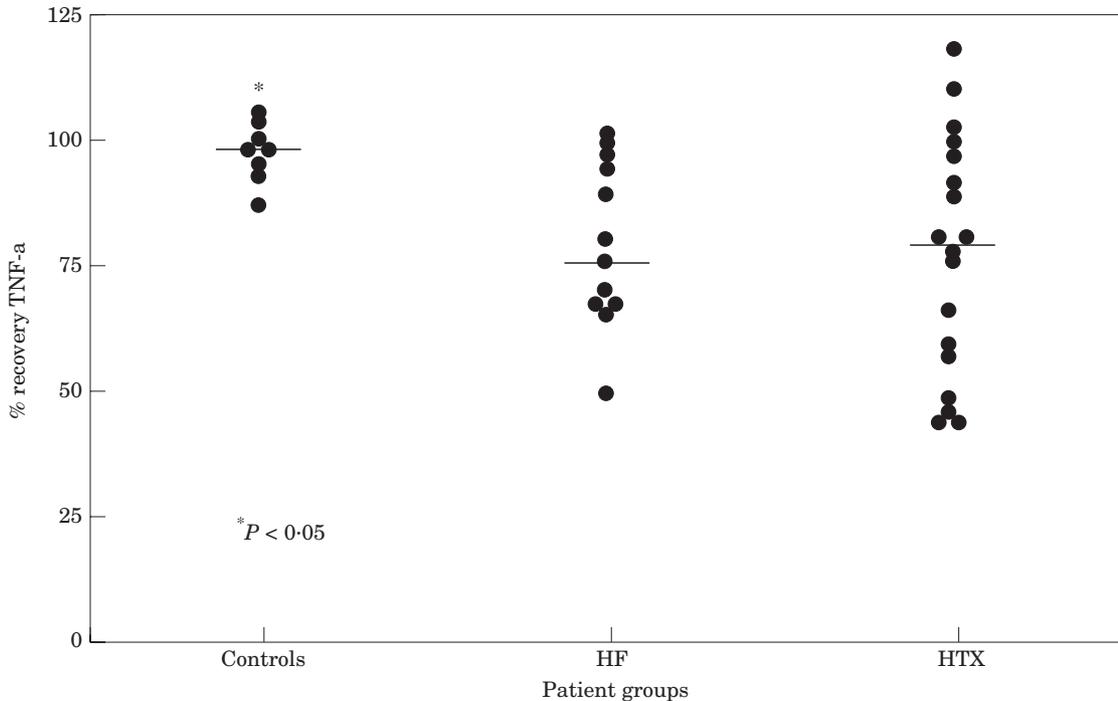
Figure 5 **TNF-α recovery, measured after incubation of 30 min of recombinant TNF-α in plasma of patients with heart failure (HF) and after heart transplantation (HTX) and control plasma, by ELISA, denoted as percentage of remaining TNF-α.**

levels[26]. After successful kidney transplantation, the TNF-α/sTNF receptor balance was only in part restored, mainly because of persistent elevation of the plasma levels of soluble TNF receptors. The reason for these high levels remains unclear. In renal transplant recipients, TNF-α production may be provided by graft-infiltrating cells to the transplanted kidney. After binding to local receptors, production of sTNF receptors occurs. In addition, impaired renal clearance, due to nephrotoxic drugs, results in high plasma levels of sTNF receptors. This 'central' cytokine concept parallels the situation in heart transplant recipients, in which TNF-α production by graft-infiltrating cells leads to high production of sTNF receptors[9]. However, a normal renal function seems a prerequisite for maintaining the physiological balance between TNF-α and its receptors.

In this study, the sTNF receptors levels showed a positive correlation with the serum creatinine both in patients with heart failure and after heart transplantation (Fig. 4(a) and 4(b)). Healthy controls reside below the regression line, suggesting that besides renal clearance other factors, for example production, plays a significant role. We found an unbalanced TNF-α system in patients with heart failure and after heart transplantation, caused by high levels of soluble TNF receptors, rather than high levels of TNF-α. The normal expression of mRNA-TNF-α by peripheral blood mononuclear cells in both patient groups suggest a non-activated 'peripheral' TNF-α system. This finding seems in contradiction with the 'peripheral' cytokine concept for the role of TNF-α in heart failure[7].

Speculation remains about the source of the high levels of the soluble TNF receptors. Production, as well as decreased renal clearance, may have contributed to the high sTNF receptor levels in our patient groups. Heart failure itself may lead to cytokine synthesis and release by inducing production of excessive catecholamines, production of angiotensin II and endothelial dysfunction, provoked by myocardial injury. After heart transplantation, TNF-α mRNA in the graft is constitutively expressed. Before, during and even after successful anti-rejection therapy with corticosteroids intragraft TNF-α mRNA expression is present[6]. This ever-present, smoldering TNF-α mRNA expression by graft infiltrating mononuclear cells, endothelium or cardiac mycocytes may be the result of ongoing allogeneic reactions in the graft. Binding to the membrane-bound TNF receptors can lead to elevated levels of soluble TNF receptors, found in peripheral blood. Nephrotoxic agents, such as cyclosporine, can diminish the renal clearance of these receptors, resulting in even higher levels. These biological active receptors can bind immunoactive TNF-α and result in decreased bioavailability of TNF-α. Complete removal of circulating TNF-α can have deleterious effects in patients, leading to high mortality from sepsis-related side-effects, as described by Fischer *et al.*[27]. In contrast, beneficial results are described in patients treated for acute rheumatoid arthritis with anti-TNF-α antibodies[28]. As TNF-α is known for its cardiodepressent properties, blocking of the TNF-α seems to favour prevention of further cardiac failure. A balance between TNF-α and

its receptors is necessary to maintain adequate immuno reactivity. Imbalance between TNF-*a* and receptors can lead to increased immunosuppression, contributing to the high incidence of infections and malignancies in these patients. In conclusion, patients with end-stage heart or renal failure and after transplantation suffer from generalized immunosuppression, in which the TNF-*a* system may play a central role. To overcome this decreased immunoreactivity, due to the imbalanced TNF-*a* system, we have to increase clearance of sTNF receptors and thus decrease TNF-*a* buffer capacity of the plasma to a level which is encountered in healthy controls.

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