The Tumor Necrosis Factor (TNF)-α system in organ failure and transplantation

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Het Tumor Necrosis Factor (TNF)-α systeem bij orgaanfalen en transplantatie

PROEFSCHRIFT

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CHAPTER 1:

General Introduction

1.1 Tumor Necrosis Factor-α

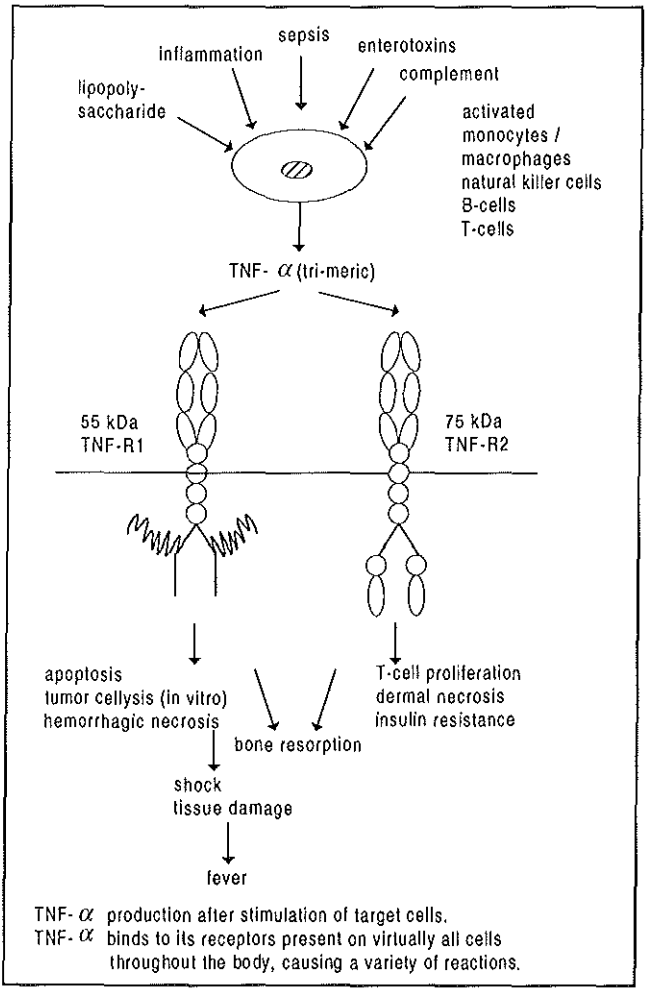
1.1.1 History of TNF-α

In the 19th century (1891) Coley observed that bacterial infections coincide with "spontaneous" regression of tumors and developed his so-called Coley's toxin, a mixture of Streptococcus pyogenes and Serratia marcescens and applied this toxin as an anticancer treatment[1]. The agent responsible for the anti-tumor effect has been identified in mice treated with Bacillus Calmette-Guérin (BCG) in combination with lipopolysaccharide, as a soluble agent, later named tumor necrosis factor (TNF)-α [2]. Tumor-necrosis factor-α (TNF-α) belongs to the family of cytokines, which are small glycoproteins (molecular weight of less than 30 kDa), produced by both hematopoeitic and non-hematopoeitic cells. The protein was isolated and cDNA was cloned in 1984[3]. In addition, it was characterized as the hormone cachectine, which suppressed the expression of lipoprotein lipase and other anabolic enzymes in fat[4]. Soon thereafter, its role as endogenous mediator in septic shock was revealed and its pro-inflammatory character was established[5-7]. Recently, the anti-tumor capacity of TNF-α has received renewed attention. Acute softening of the tumor, hemorrhagic necrosis and occlusion of the neo-vasculature leading to tumor necrosis revealed a possible object to anticancer therapy[8,9]. However, severe toxicity was encountered in phase I/II clinical trials, in which progressive heart failure was a prominent feature[10], which excludes systemic therapy. Nevertheless, local institution of TNF-α by isolated limb perfusion, shows encouraging results and may predict a new era of TNF-α as anti-tumor regimen[8,9,11].

1.1.2 Production, working mechanisms and function of TNF-α

TNF-α, a pro-inflammatory cytokine, is predominantly produced by activated cells of the immune system: monocytes, macrophages, natural killer (NK) cells, B-cells and T-cells. TNF-α binds to receptors present on virtually all cells throughout the body,

except on red blood cells. Conditions, known to induce TNF-α production are inflammation, fever, acute phase reaction, and sepsis. Monocyte activation by endotoxins, like lipopolysaccharides (LPS), enterotoxins, toxic shock sydrome toxin, mycobacterial wall products (lipoarabinomannan), antibodies to monocytes (CD14), and products of complement may result in a peak outburst of TNF-α[12]. Lymphocyte activation by antigenic or mitogenic stimuli also results in TNF-α production[13,14].



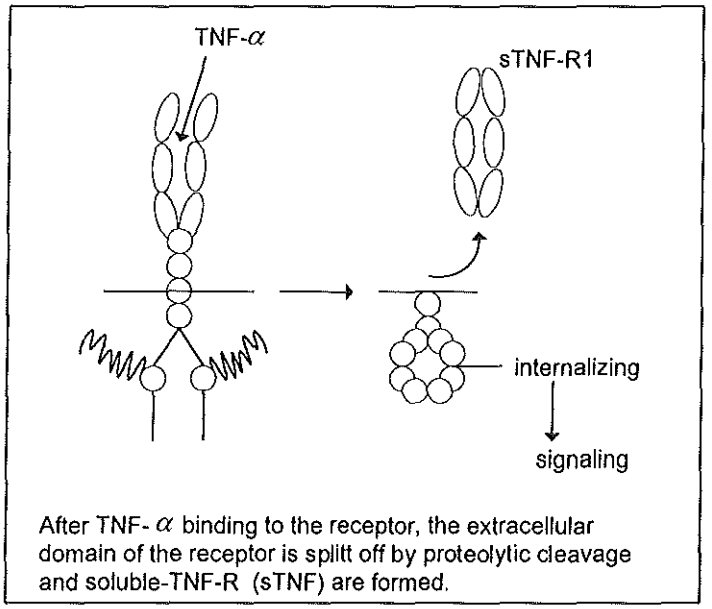
TNF- α production after stimulation of target cells.
TNF- α binds to its receptors present on virtually all cells throughout the body, causing a variety of reactions.

TNF-α appeared to be a 17kDa glycoprotein, which in its active form consists of 3 molecules. This trimer is the biological active form of TNF-α and binds to the membrane-bound TNF receptor complex.

The affinity of cytokines to their specific receptors is very strong. The dissociation constants (Kd) for TNF-α to its receptors are 2-5 x 10^{-10} and 3-7 x 10^{-10}M[15] and result in biological activity at very low concentration levels[4]. For TNF-α two distinctive receptors have been described: a 55 kDa TNF-R (TNF-R1) and a 75 kDa TNF-R (TNF-R2) (see figure).

Biological activity mediated by TNF-R1 is distinct from activity mediated by TNF-R2. Cytotoxicity and apoptosis (programmed cell death) is predominantly mediated by TNF-R1, while cytotoxicity, and T- and B-cell proliferation induction are mainly conducted by TNF-R2[16-18].

However, as result of pleiomorfism, the function of R1 can partially be inhibited by affecting R2[19]. Rink reports the differentiation between the function of R1 and R2, however conflicting data are present concerning the clearness of the distinction between functions of R1 and R2 as reported by others[20]. After binding of TNF-α to its receptor, proteolytic cleavage of the extracellular domains result in the production of soluble receptors, sTNF-R1 and sTNF-R2 (see figure). These soluble receptors can bind 1-3 circulating TNF molecules and act as a pool of TNF-α[4].



After TNF- α binding to the receptor, the extracellular domain of the receptor is splitt off by proteolytic cleavage and soluble-TNF-R (sTNF) are formed.

TNF-α, if released systemically in large amounts all at once, activates neutrophils, modifies anticoagulant properties of the endothelium, and induces the release of other inflammatory cytokines, such as IL-1β and IL-6. These effects can culminate in cardiovascular collaps and shock. On the contrary, chronic, low-level production of TNF-α may contribute to inflammatory responses in autoimmune diseases like rheumatoid arthritis, morbus Crohn, but also attributes to bone resorption, fever, anemia and wasting[4]. Direct cytotoxic effects of TNF-α on endothelial cells result in upregulation of major histocompatibility complex (MHC) class I and II molecules, adhesion molecules, and chemoattraction of polymorphonuclear cells, which, after TNF-α activation release free radicals and thereby damage the endothelium[21].

1.2 TNF-α and its role in the immune reponse

1.2.1 Introduction of the immune system

The immune system has evolved to protect us from pathogens. Specific immune responses occur by different stimuli (antigens), e.g. microbiological agents,

alloantigens or auto-immune antigens. Antigens are processed by antigen presenting cells, e.g. monocytes, macrophages and polymorphonuclear neutrophils, which in turn present the antigens to specific lymphocytes that recognize the antigen, resulting in initiation of the immune response and elimination of the pathogen. In this process lymphocytes have specific functions. Helper T-cells coordinate the immune response by direct cell-cell interactions and the release of cytokines, which help B-lymphocytes to secrete antibodies (humoral immune response), activate T-lymphocytes to proliferate, and to kill the target cell (cytotoxic T-cells).
This specific immune response consists of 3 different distinct signals. Signal 1 refers to the antigen presentation, in which the APC process the antigen and presents it in its MHC class II molecule to the T-cell receptor (TCR), present on T-cells[22]. Signal 2 is named the co-stimulatory pathway. Interaction of the T-cells with the APC needs this co-stimulation in order to become activated. There are different co-stimulatory pathways, in which each molecule on the T-cell interacts with its ligand on the APC. Well-known combinations are the CD28/$B7_1$ and the CD40/CD40L interaction. Signal 3 refers to the production of cytokines, like IL-1, IL-6 and TNF-α.
After signal 1 to 3 the T-cell is activated and IL-2-production is started. This IL-2 production results in proliferation and differentiation of helper T-cells and cytotoxic T-cells. Furthermore, IL-2 augments the cytolytic activity of NK cells, is involved in programmed cell death of activated T-cells, and promotes the synthesis of immune globulins by B-cells[23].

1.2.2 TNF-α and the immune response

In 1987 Waage first demonstrated high levels of circulating TNF-α in patients with severe meningococcal disease[24]. Positive correlation between TNF-α levels and clinical outcome has been found[25]. In an immune response the high levels of TNF-α are predominantly produced by monocytes, but also T-cells and natural killer cells produce TNF-α, upon stimulation. The production of TNF-α is strongly induced by lipopolysaccharides. Cytokines, e.g. IL-15 and IFN-γ, mediate TNF-α production by T-cells and monocytes[26,27]. Synchronous to the production of TNF-α is the production of the soluble TNF receptors, sTNF-R1 and sTNF-R2.
Auto-immune inflammatory diseases, as well as bacterial induced inflammation is largely conducted by production of TNF-α at the site of inflammation[28,29]. The levels of TNF-α reflect the disease activity in morbus Crohn[30,31]. The central role of TNF-α in the immune response has been accepted, and has resulted in the development of anti-

TNF therapy, e.g. in morbus Crohn[32,33], sepsis[34] and rheumatoid arthritis[35,36]. Benificial effects of blocking TNF-α by antagonists is described in rheumatoid arthritis[35], but complete removal of TNF-α has deleterious effects in sepsis[34].

1.3 TNF-α in renal failure and dialysis

TNF-α is thought to play an important role in progressive renal failure[37-39]. High expression levels of TNF-α are found in kidneys of patients with glomerulonefritis, i.e. membranoproliferative or mesangial proliferative[40]. In IgA glomerulopathies, high circulating levels of TNF-α are found in presence of macroscopic hematuria[41]. In non-active or non-proliferative or non-hematuric glomerulopathies TNF-α levels are within the normal range. TNF-α is produced by cells of the affected kidney[42,43], but also by peripheral blood mononuclear cells (PBMC). For instance PBMC from patients with glomerulonefritis produced higher amounts of TNF-α than PBMC from healthy controls[44,45]. Mechanisms leading to progressive renal failure involve actions of angiotensin-converting enzyme (ACE) and therefore ACE-inhibitors may be of benefit in those patients possibly by influencing TNF-α production[46,47]. Increased TNF-α levels coincide with the malnourishment, which is often encountered in severe renal failure[48], and may be even the cause of uremia related cachexia.

Another field in which TNF-α is thought to play a key-role is in hemodialysis related disease. Hemodialysis procedures are frequently followed by fatique, fever, malaise and anorexia, while the hemodialysis-related hypotension can be a problem in some patients resulting in inadequate dialysis sessions[49].

In 1983 the "interleukine hypothesis" was put forward: cytokines produced during hemodialysis are involved in the processes leading to hypotension, fever, and other acute phase responses observed in patients on dialysis[49]. Due to continuous blood-membrane contact in patients on dialysis, monocytes become activated, reflected by high levels of TNF-α in patients on renal replacement therapy[50,51]. TNF-α protein, in contrast to the soluble receptors, sTNF-R1 and sTNF-R2, is not detectable in plasma of healthy subjects[50]. However, when chronic renal failure occurs, even in mild forms (creatinine clearance 40-80 ml/min), elevated TNF-α plasma levels can be found[50]. The plasma concentration of TNF-α shows a positive relation with progressive renal failure. In patients on renal replacement therapy, peritoneal dialysis (CAPD) or hemodialysis (HD) higher plasma levels of TNF-α are found. The presence of free plasma TNF-α however, is not constitutively observed by all investigators[52-54].

Furthermore, TNF-α could play a role in the immune dysfunction seen in these patients. Increased susceptibility to infections, malignancy, and artherosclerosis are often found in patients on chronic hemodialysis. TNF-α, but also IL-1β, two pro-inflammatory cytokines, are likely to mediate these dialysis-related symptoms[49,55-66]. The activated state of the monocytes contrasts with the increased immune incompetence of patients on HD. The role the soluble TNF receptors as possible inhibitors of the biological activity of TNF-α or as pool of bioavailable TNF-α is still not completely clarified. The balance between TNF-α and its soluble receptors may be more important than the exact levels of the proteins itself[67,68]. Except TNF-α, many other cytokines have been studied in patients on HD. *In vivo* as well as *in vitro* production of IL-2, IL-6, IL-8, IL-10 and IL-12 by different cell types upon different stimuli have been described[61,69-71]. Overall, it seems that after *in vitro* stimulation, monocytes are capable of producing adequate amounts of multiple cytokines[64,72]. Hemodialysis procedures are supposed to induce cytokine production, resulting in dialysis related morbidity. Moreover, HD patients show no adequate immune reponse on T-cell dependent antigens, i.e. hepatitis B vaccination in contrast to patients on peritoneal dialysis[73,74]. The HD procedure, itself, seems to result in an inadequate immune response. Whether the defects in the immune response are at levels of APC, at T-cell levels or at the level of interaction between the APC and T-cells is not yet clear. The APC are activated and produce cytokines[50]. The T-cell function in uremia and hemodialysis is extensively described by many authors. Normal, as well as intrinsic defects in T-cell are found[75-77]. However, initiation of hemodialysis treatment leads to improvement of T-cell and immune function, suggestive for an defective T-cell function[62,78]. TNF-α and T-cell function are also clearly related, as Tartaglia described[19]. Therefore, TNF-α may play a central and pivotal role in the disturbed immune response in HD patients, but its role on the functions of the immune system in patients on renal replacement therapy is all but clear and can be described as pleiomorf as the cytokine itself.

Illustrative is the appiclation of continuous arterio-venous or veno-venous hemodialysis procedures (CAVHD, CVVH) in case of severe sepsis and hemodynamic instability as therapy of choice, in order to clear the overshoot of pro-inflammatory cytokines, like TNF-α, IL-1β and IL-6[79-83].

1.4 TNF-α and its role in heart failure

Patients with chronic heart failure can suffer from cardiac cachexia that may be due to activation of the immune system[84]. Increased circulating levels of TNF-α in patients with progressive heart failure are frequently described[86-91]. Furthermore increased expression of TNF-α has been found in cardiac tissue of explanted heart from patients undergoing cardiac transplantation[85]. Besides the proinflammatory properties, TNF-α is known for its direct toxic effects on cardiomyocytes resulting in LV dysfunction, pulmonary edema, LV remodelling, and cardiomyopathy[92,93]. These cardio-depressent properties of TNF-α were discovered after the introduction of TNF-α as anti-tumor therapy. A high incidence of severe dilated cardiomyopathy was encountered in TNF-α treated patients[10]. Animal studies have shown that transgenic mice that overexpress TNF-α in the cardiac compartment develop progressive left ventricular (LV) dilatation and LV dysfunction[91]. The origin of these high levels of TNF-α is a point of discussion. In failing hearts increased mRNA expression as well as cytosolic TNF-α is found, however, no correlation between intracardiac TNF-α and serum levels was established[85]. On the other hand, heart failure may cause edema of the gut wall, which subsequently may result in altered gut permeability and fascilitate endotoxin release and TNF-α production by immune competent cells[84]. Also neuro-endocrine disorders, with stimulation of the sympatic nervous system, the renin-angiotensin-aldosteron axis and the natriuretic peptide system, which in turn can stimulate TNF-α production, have been proposed[94]. High levels of TNF-α are associated with progressive heart failure. Recovering from severe heart failure by insertion of left ventricular device, coincides with a decrease of TNF-α[95]. Also studies with TNF-α antagonists treatment in patients with severe heart failure improved the function of the failing heart, without serious side-effects[96]. The favorable effects of continuous hemodialysis (CAVHD, CVVH) in patients undergoing cardiac surgery is thought to be the result of clearance of the pro-inflammatory cytokine TNF-α[97-99]. High levels of TNF-α are associated with increased TNF-R expression on monocytes, endothelial cells, and on cardiomyocytes[85,100]. Shedding of the TNF-R results in production of soluble TNF-R in peripheral blood. These sTNF-R provide a pool for TNF-α by binding the active, trimeric TNF-α molecule and prevent dissociation of TNF-α to inactive monomers[87]. As sTNF-R are metabolized by renal clearance it is obvious that sTNF-R show a positive correlation with serum creatinine[101]. Serum levels of sTNF-R predict outcome after cardiac surgery. Whether this reflects the better renal function or a larger pool of active TNF-α remains unclear[102]. The central role of TNF-α in heart failure is well established. On

the longterm the effects of a continuously activated immune system with increased TNF-α levels have to be evaluated, in relation to the overall immune competence of patients with heart failure.

1.5 TNF-α in organ transplantation

1.5.1. TNF-α in renal transplantation

Much attention has been focused on the involvement of specific cytokines after renal transplantation[103]. In particular, their involvement in graft rejection and tolerance has been evaluated in the clinical setting and in experimental models[104-109]. TNF-α plays a role in graft rejection. A significant increase of TNF-α plasma levels was seen a few days before allograft rejection[110]. Dörge et al measured TNF-α and the soluble receptors R1 and R2 before and daily after renal transplantation. These authors clearly showed an increase of TNF-α during acute allograft rejection[111]. Except measurements in peripheral blood, intragraft detection of specific cytokine patterns have shown to be correlated with rejection. Especially, increased mRNA expression of IL-2 and IL-2R was positively related to subsequent episodes of acute rejection[112]. Messenger RNA expression of TNF-α, IL-1β and IL-10 were also found during acute rejection, but were not predictive[112,113]. Cytokine production is genetically determined and some authors described the predictive value of TNF-α gene polymorphisms and the occurrence of renal allograft rejection[114,115]. Especially the TNF-α high-producer genotype of the recipient was positively correlated with multiple rejection episodes[115]. As the role of TNF-α in rejection is established, inhibition of the TNF-α effects as anti-rejection therapy can be performed, using recombinant human TNF receptors (TNF-R). Eason et al described encouraging results in delay of rejection when TNF-R was given alone, or in combination with CsA, in non-human primates[116]. Sabatine et al performed a study with sTNF-R secreting tumor allografts which resulted in prolongation of the transplanted graft without rejection, suggestive for immunosuppressant properties of sTNF-R[117]. It is clear that TNF-α and the receptors R1 and R2 play a role in the immune response after renal transplantation. The production by activated macrophages and T-lymphocytes in the allograft as result of allograft rejection may be the source of the increased TNF-α levels. The elevated levels of sTNF receptors may result from increased shedding and thereby reflect the activity of the TNF-α system after renal transplantation. A strong correlation between these levels of the soluble receptors and

serum creatinine has been described[105]. Therefore, measuring TNF-α and the soluble receptors after transplantation may provide a non-invasive diagnostic tool for rejection.

1.5.2. TNF-α in heart transplantation

After heart transplantation elevated levels of circulating TNF-α, and the receptors TNF-R1 and TNF-R2 may predict severe humoral and cellular rejections[118]. But, when the TNF-α levels were measured in coronary sinus serum, no relation with histological signs of rejection was found[119]. These conflicting results are in part due to the different circumstances under which cytokine levels were measured. It appeared that elevated TNF-α levels, but also IL-6 levels, were found after anti thymocyt globulin (ATG)-induction therapy[120]. In contrast, Deng et al described a positive correlation between serum levels of IL-6, TNF-α and sIL-2R and hemodynamic (pulmonary wedge pressure, pulmonary arterial pressure, right atrial pressure and heart rate) and ultrasound (isovolumic relaxation, fractional shortening) parameters in cardiac allograft recipients[121]. When sequential blood samples were examined, TNF-α serum level correlated well with the findings in endomyocardial biopsies[118]. Cytokine measurements in peripheral blood may not entirely reflect the rejection process in the transplanted heart[122]. Intragraft expression of cytokines at mRNA levels has been frequently described[123,124]. But again, conflicting data are presented. Positive, weak and no relation between TNF-α and rejection are described[124-126]. However, mRNA expression is genetically regulated and gene polymorphisms are found for multiple cytokines, including TNF-α. Again, in comparison to renal transplant recipients, the high TNF-α producer genotype of the recipient appeared to be more vulnerable to acute rejection[127]. Furthermore, Baan et al found an everpresent TNF-α mRNA expression in endomyocardial biopsies, but the expression level was clearly related to rejection, since it significantly decreased after anti-rejection therapy[125]. These data suggests that TNF-α is involved in rejection processes after clinical heart transplantation and may provide another diagnostic tool. However, the relation between TNF-α and cardiac allograft function has still to be evaluated.

1.6 Aim of this thesis

We analyzed in detail the TNF-α system during organ failure and organ replacement therapy in an attempt to assess the role of TNF-α in the pathogenesis of the immune compromised status of patients with these conditions.

In this respect we measured mRNA expression, protein production, receptor expression, soluble receptor levels, serum buffer capacity and the TNF-α induced cell proliferation in patients with renal or heart failure, in patients on renal replacement therapy (peritoneal dialysis or hemodialysis), and in patients after kidney or cardiac transplantation. To determine whether the severity of renal failure or the mode of renal replacement therapy result in increasing abnormalities of cytokine systems, the TNF-α system was evaluated at various levels of renal insufficiency: pre-dialysis end-stage renal failure (ESRF), on peritoneal dialysis (CAPD) and hemodialysis (HD) (*chapter 2*).

TNF-receptors are expressed by both peripheral monocytes and T-lymphocytes. *Chapter 3* describes a quantitative flow cytometric analysis to assess the activation status of the TNF-α and IL-2 system at the single cell level in patients with ESRF, on CAPD and HD.

To test whether an activated TNF-α system in patients on hemodialysis results in impaired T-cell response, various parameters of the TNF-α system (Antigen Presenting Cell) and IL-2 system (T-cells) were analyzed (*chapter 4*), while the cytokine-driven (TNF-α, IL-2) proliferation of T-cells was measured to find out whether the immune dysfunction was due to an intrinsic T-cell defect (*chapter 5*).

In *chapter 6* the TNF-α system after renal transplantation is described. We performed serial measurements of serum and urine samples, to study the clearance of the soluble receptors, directly after transplantation and at 1 year. These measurements were performed after living (un-)related renal transplantation in order to prevent other factors, which may influence the TNF-α levels, e.g. delayed graft function.

In *chapter 7* the activity of the TNF-α system on mRNA level, plasma protein, and soluble TNF-receptors are described in patients with heart failure and after heart transplantation.

Membrane receptor expression on target cells may reflect the activity of cytokine systems. In the immune response the cytokines TNF-α and IL-2 play a pivotal role. The impaired immune competence seen after transplantation might not only be the consequence of immunosuppressive therapy, but also be the result of unbalanced cytokine systems. To assess the activition status of the cytokine systems TNF-α and IL-2, the receptor expression of these cytokine systems on peripheral monocytes and lymphocytes is measured, using the quatitative flow cytometry in *chapter 8.*

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CHAPTER 2

TNF-α: mRNA, plasma protein levels and soluble receptors in patients on chronic hemodialysis, on CAPD and with end-stage renal failure

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Abstract

Patients on hemodialysis suffer from an impaired immunity against infectious agents, hyporesponsiveness to vaccination and are prone to develop malignancies. This clinical state of immunoincompetence may be due to a unbalance in their defense mechanisms in which TNF-α and its soluble receptors 1 and 2 play a central role. We measured, with double-sandwich ELISA, the levels of TNF-α and the soluble TNF-receptors in peripheral blood of patients on chronic intermittent hemodialysis (CIHD), on peritoneal dialysis (CAPD) and pre-dialysis end-stage renal failure (ESRF). Using Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) analysis, we quantified the amount of TNF-α mRNA in peripheral blood mononuclear cells (PBMC) obtained from these patient groups. In none of the patient groups elevated levels of TNF-α were detected with ELISA, while high levels of soluble TNF-receptors were present in ESRF, CAPD and CIHD patients. This may be the result of an activated TNF-α system or due to their impaired renal clearance. TNF-α mRNA level was elevated in CIHD patients compared to ESRF and CAPD patients or healthy controls. This suggests that only during chronic HD the TNF-α system is activated. High levels of sTNFR, found in ESRF or CAPD patients do not reflect activation of TNF-α system, but are the result of impaired renal clearance of the receptors. Indeed, we found a strong linear correlation between the levels of sTNF receptors and renal function. Nevertheless, these high levels of sTNF-receptors are biological active, as they were able to bind active TNF-α up to 75% (range 46-83%) and thus inhibit the bioactivity and bioavailability of produced TNF-α. This may play a role in the immunoincompetence of these patients.

Key words: TNF-α, ESRF, renal replacement therapy, immunoincompetence

Introduction

Patients on chronic hemodialysis are susceptible to infections and malignancies and show a decreased responsiveness in delayed hypersensitivity tests and defective antibody production to T-cell dependent antigens such as influenza and hepatitis B vaccine [Descamps-Latscha 1993, Girndt et al 1993, Girndt et al 1995]. This seems to contrast with the activation of the macrophage/monocyte system, as can be concluded from their increased cytokine production [Descamps-Latscha et al 1995, Descamps-Latscha and Jungers 1996, Donati et al 1992]. Activation of the monocytes may be due to the blood-membrane interaction, as the production level of these inflammatory cytokines reflects the biocompatibility of the dialyser membrane [Lin et al 1996, Kino

et al 1995, Lonneman et al 1990, Niwa et al 1995]. Cytokine production alone, however, can not predict the final outcome of an immune process, as both bioavailability and bioactivity of these cytokines are highly dependent on the presence of their antagonists [Bazzoni and Beutler 1995, Bazzoni and Beutler 1996, Douvdedani et al 1996]. For TNF-α the antagonists are the soluble TNF-receptors (sTNF), which are the split-products of the membrane-bound TNF-receptors R1 and R2. The sTNF-receptors are formed by proteolytic cleavage of the membrane-bound TNF-receptors after binding with TNF-α. In plasma the sTNF-receptors bind 1-3 TNF-α molecules with an affinity (K_d) of approximately 0.5 nM (sTNF-R1) and 0.1 nM (sTNF-R2) [Meager et al 1996]. However, not all sTNF-receptor binding sites are occupied, leaving room for a certain buffer capacity for TNF-α [Bazzoni and Beutler 1996]. *In vitro* studies revealed that the cytotoxic and inflammatory actions of TNF-α can be blocked by an excess of sTNF-R1 and sTNF-R2 [Terlizzese et al 1996]. It has been shown that the TNF-α/sTNFR-system is severely affected in patients with end-stage renal failure and in patients on renal replacement therapy [Pereira et al 1994, Noronha et al 1995]. Although free TNF-α plasma levels are not consistently found in HD patients [Lin et al 1996, Lonneman et al 1996], high levels of several cytokines, including TNF-α, can be found. These cytokines are mainly produced by peripheral blood mononuclear cells upon stimulation [Roccatello et al 1993, Engelberts et al 1994]. The increased levels of sTNF-receptors may be the result of activation of the TNF-α system, but also impaired renal clearance [Floege and Gröne 1995, Halwachs et al 1994, Lambert et al 1994]. Commercially available ELISA kits give variable results, due to differences in affinity for bound TNF-α to circulating antagonists or TNF-inhibiting proteins [Kreuzer et al 1996]. Measurements of mRNA expression by peripheral blood mononuclear cells (PBMC) for TNF-α, using RT-PCR is a semi-quantitative method determing the level of activation of the TNF-α system. In the present study, we assessed the activity of the TNF-α system by measuring free TNF-α protein plasma levels, sTNF-receptors, R1 and R2, and the TNF-α mRNA expression by PBMC. Moreover, we tried to determine the biological activity of soluble receptors by measuring the TNF-α binding capacity of patient plasma. Pre-dialysis ESRF patients were compared with patients on chronic intermittent hemodialysis (CIHD) and continuous ambulantory peritoneal dialysis (CAPD). Healthy laboratory personel served as controls.

Material and Methods

Patients

A total number of 48 subjects were analyzed. We studied 15 CIHD patients, who were on hemodialysis for a mean period of 35 months (range 12-59 months, median 38 months). All patients were in stable hemodynamic condition during the dialysis procedure with no signs of infection or malignancy. They were dialysed 2 (n=8) or 3 (n=7) times a week for 4-5 hours, in order to reach a Kt/V of approximately 1.35. The dialyser membranes used in this study were Polysulphone (F60 Fresenius, AG, Bad Homburg, Germany, n=9) and Hemophane (MA-12H, Kawasumi Laboratory Inc., Minamiohi Shinagawa, Tokyo, Japan, n=6). All patients were on bicarbonate dialysate. The dialysate was routinely cultered, no periods of contamination were encountered. Culture results were $< 10^{3}$ micro-organisms per ml. Stable CAPD patients (n=10), using Baxter twinbag system (Kt/V=1.5), and patients with ESRF (n=11) were used as control patient groups, while 12 healthy subjects served as controls. Causes of renal failure were hypertension (n=9), membranous glomerulonephritis (n=11), Wegener's granulomatosis (n=1), focal segmental glomerulosclerosis (n=2), polycystic kidney disease (n=4), amyloidosis (n=1), reflux nephropathy (n=3) and IgA nephropathy (n=1) and unknown (n=3). Causes of renal failure were equally represented in the patient groups. Most patients were on anti-hypertensive drugs: Calcium-entry blockers, ACE-inhibition and β-blockers, while all patients on renal replacement therapy (CAPD and CIHD) were on subcutaneous recombinant erythropoietin therapy.

Sample preparation

Blood samples were collected in pyrogen-free tubes containing EDTA in a final concentration of 1 mg/ml at the start of the hemodialysis-procedure. In patients with ESRF and CAPD the blood samples were collected during outdoor clinic control visits. The samples were immediately centrifugated, plasma and cell-fractions were separated and the plasma was stored at -80 °C. For the isolation of PBMC the buffy coat was diluted in phosphate-buffered saline (PBS) solution and layered on a Ficoll-Isopaque gradient (δ = 1.077). After centrifugation, the peripheral mononuclear cells (PBMC) were removed from the interface, and washed twice with ice-cold PBS. Immediately following procurement, 2 x 10^{6} PBMC were snap-frozen in liquid nitrogen and stored at -80 °C for RT-PCR analysis.

Isolation of mRNA and cDNA reaction

Total mRNA was extracted from PBMC by a modification of the guanidinium method previous described by our group [Baan et al 1994]. Cells were homogenized in 500 µl 4 mol/l 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. The total mRNA was precipitated with 600 µl 2-propanolol and 35 µl 3 mol/l sodium-acetate (pH 5.2) at -20 °C for 18 hours. The precipitates were pelleted at 10.000xg at 4 °C and washed with 500 µl ice-cold 80% ethanol. Air-dried pellets were resuspended in 50 µl diethylpyrocarbonate treated H_2O. mRNA was denaturated for 5 minutes 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, WI) and transcribed with 1000 U Moloney murine leukemia virus reverse transcriptase (Gibco-BRL, Gaithersburg, MD) at 42 °C for 90 minutes in a total volume of 100 µl.

Competitive template RT-PCR

Sequence specific primers were used for amplification of the human TNF-α gene. TNF-α sense primer: 5' GAG TGA CAA GCC TGT AGC CCA TGT TGT AGC A 3', and TNF-α anti-sense primer: 5' GCA ATG ATC CCA AAG TAG ACC TGC CCA GAC T 3'. PCR primers detecting transcripts for the human house-keeping gene, keratin, were used as an internal control to monitor mRNA extraction and cDNA amplification [Baan et al 1994]. To estimate the relative initial amount of functional TNF-α mRNA in PBMC a competitive RT-PCR assay was used and comparison was made against the house-keeping keratin gene. The latter gene is assumed to be expressed at a constant level in PBMC. Five µl cDNA sample and 5 µl of gene specific competitive templates were added to 90 µl PCR mixture containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each 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 in different dilutions to constant amounts of sample cDNA for competitive co-amplification 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 (TNF-α: 444 bp. versus 326 bp., Fig.1A). For

keratin: the target 218 bp. and internal control 160 bp. 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, NJ) under the following conditions.

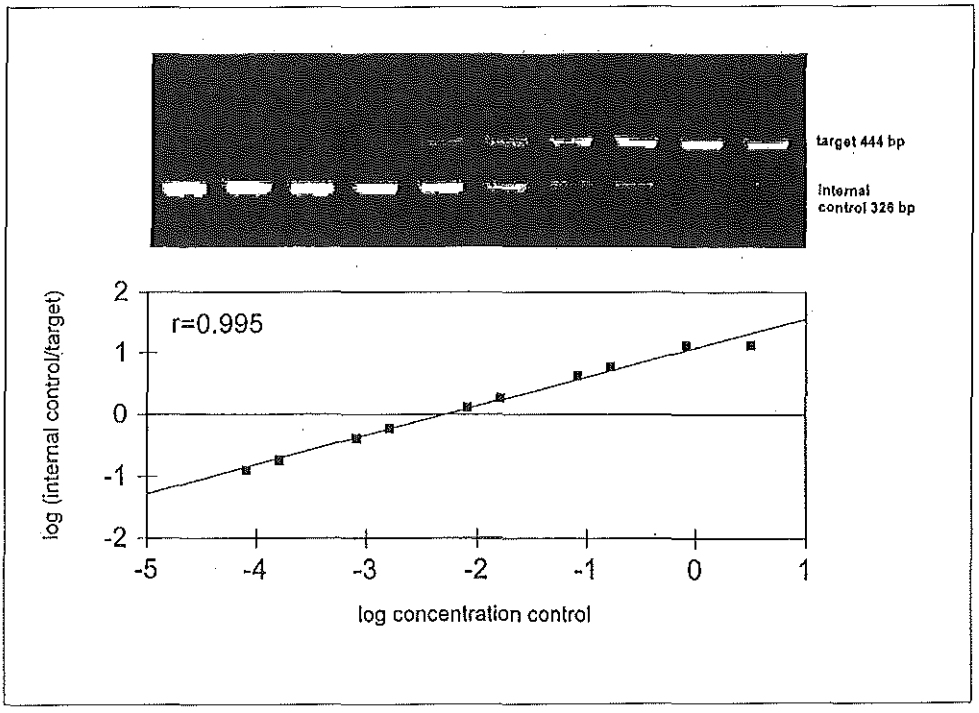

Figure 1A: (upper panel)
After electrophoresis, the ethidium bromide stained agarose gel shows 2 PCR products per lane representing target (444 bp) and internal standard (326 bp)
Figure 1B: (lower panel)
The log of the ratio amplified TNF-α to competitor TNF-α is graphed as a function of the log of the known amount of competitor added to PCR. At the point where target cDNA and internal control are in equivalence (ratio=1), the starting concentration of TNF-α cDNA prior to PCR is equal to the known starting concentration of the competing internal control.

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% phytohemagglutinin (PHA)-M (Difco, Detroit, MI) for 4 hours 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 no-template reaction. Following PCR, 16 µl PCR product was analyzed by gel electrophoresis and the amount of products by the internal control and targets were determined for each individual 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). 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 TNF-α and keratin cDNA prior to PCR is assumed to be equal to the known starting concentration of the competing internal control (Fig.1B). The relative concentration of TNF-α transcripts were divided by the relative concentration of keratin. This represents the amount of TNF-α mRNA transcripts corrected for the amount of mRNA used for reverse transcription and the efficacy of each reaction.

TNF-α and soluble TNF-receptors by ELISA

In a preliminary study we compared 4 commercially available ELISA kits. All kits are mostly based on mouse-antihuman TNF-α and biotinylated mouse-antihuman TNF-α. The Genzyme duo set uses biotinylated rabbit-antihuman TNF-α in step 2 of the double sandwich ELISA, which is probably the reason for their lowest detection limits (1.4 pg/ml), compared to the 3 other ELISA tests (detection limits: 4.1-10.0 pg/ml). Medgenix and Pharmingen use specific clones of Mabs. In this study, we used the ELISA kit (Central Laboratory of Blood Bank, Amsterdam, The Netherlands), which was performed following the manufacturers instructions. The detection limit for TNF-α was 10.0 pg/ml and the coëfficient of variation was less than 10%. The standard curve is steep, especially in the measurement range. The sTNF-receptors R1 and R2 were measured by ELISA (R&D Systems Europe, Abington, United Kingdom). The detection limit was 15 pg/ml for both sTNF-R1 and -R2. The coefficient of variation was <5% (sTNF-R1) and <10% (sTNF-R2).

Biological activity (the ability to bind circulating TNF-α, *in vitro*) of the soluble TNF-receptors was determined after 30 minutes incubation of patient plasma with recombinant human TNF-α (final concentration: 300 pg/ml). After incubation, the concentration of free TNF-α was measured using the above mentioned ELISA

technique. A standard curve was constructed using different concentrations of recombinant human TNF-α. The TNF-α concentration after incubation was calculated by extrapolation to the standard curve. The TNF-α recovery is the residual TNF-α concentration (after incubation) divided by the start concentration (TNF-α concentration in patients plasma) x 100%.

Statistics

The results are given as data and median with range or mean with standard deviation whenever appropriate. Mann Whitney U test was used to compare medians. The results of sTNF-receptors in plasma were evaluated and compared using the ANOVA. Results of the TNF-α recovery are evaluated by the Dunn's multiple comparison test. P-values of < 0.05 were considered to be significant.

Results

All patients were in good clinical condition at time of blood sample collection. Patients on CIHD were stable during the dialysis procedure. There were no infections in the week before, at time of, or in the week after collection of the bloodsamples. Patients on CAPD did not suffer from peritonitis in the period of blood sample collection. Patients with ESRF were in stable clinical condition and did not start renal replacement therapy within several weeks after blood sample collection. Medication was continued in all patients.

TNF-α mRNA expression was detectable in all PBMC samples, including those of healthy controls. Therefore, we performed quantitative competitive template RT-PCR analysis in an attempt to detect differences in TNF-α mRNA levels between these four groups. Using the competitive template RT-PCR, we found no statistical difference among the groups in expression of the positive control housekeeping gene, keratin. This indicates that the integrity of the mRNA for the three patient groups and the controls is 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 PBMC from patients on CIHD were significantly higher than in healthy controls (median TNF/keratin ratio: 47.541 vs 528, $p < .0001$). These results show that chronic HD specifically activates the TNF-α

system. Levels of mRNA-TNF-α in PBMC from patients on CAPD or with ESRF were in the same range as healthy controls (Fig 2).

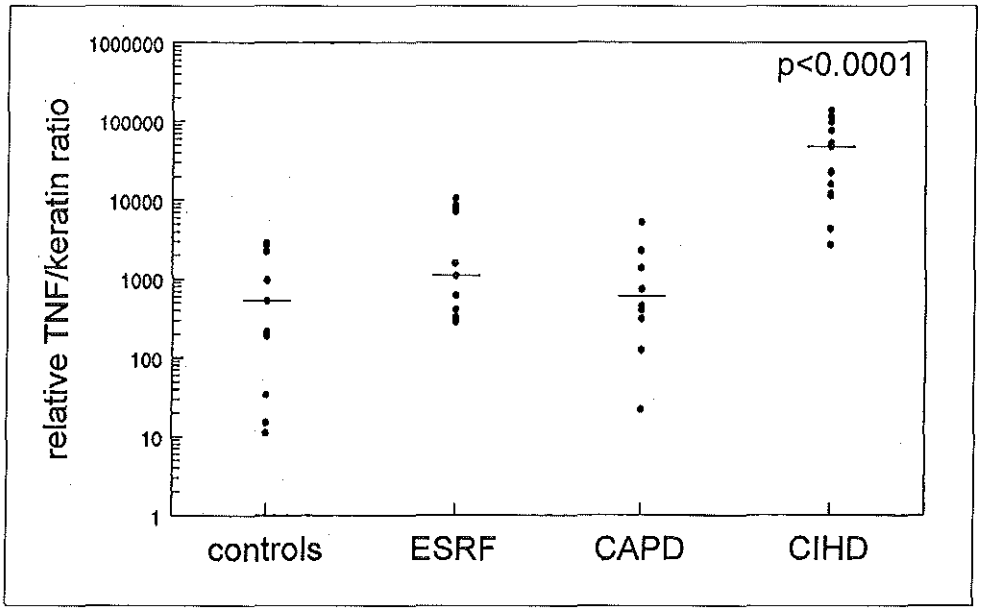

Figure 2:
Quantitative mRNA expression for TNF-α measured by RT-PCR, denoted as TNF/keratin ratio, with keratin as housekeeping gene in different patients groups

In spite of the high levels of mRNA expression of TNF-α, plasma levels of TNF-α were hardly detectable with ELISA in plasma obtained from both healthy controls and patients. To evaluate the influence of the dialysis procedure, we measured TNF-α concentrations at start of the dialysis period, after 30 minutes and at the end of the dialysis procedure. During the dialysis period no increase in TNF-α plasma protein concentrations was seen. Furthermore, no difference in TNF-α mRNA expression or TNF-α plasma protein concentrations were found in patients dialyzed with the polysulphone or hemophane membrane, indicating no difference in activation between the two membranes (data not shown). In contrast, sTNF-R1 and -R2 levels were significantly higher in ESRF, CAPD and CIHD-patients plasma compared to healthy controls (Table 1).

Table 1:
Patient characteristics and levels of TNF-α and the soluble TNF-receptors in patients with end-stage renal failure and on renal replacement therapy

	Controls (n=12)	ESRF (n=11)	CAPD (n=10)	CIHD (n=15)
M/F	4/8	5/6	8/2	6/9
Age[yrs]	30 (26-54)	46 (21-82)	45 (25-70)	63 (33-83)
Creat. [μmol/l]	< 100	552 (340-735)	1031 (586-1290)	985 (706-1325)
TNF-α [pg/ml]	<10	<10	<10	<10
sTNF-R1 [ng/ml]	0.6 ± 0.2	8.2 ± 2.5 [a]	17.1 ± 4.9 [a]	15.9 ± 4.2 [a b]
sTNF-R2 [ng/ml]	1.7 ± 0.5	14.8 ± 6.6 [a]	19.4 ± 5.0 [a]	21.3 ± 3.7 [a b]

Age: median (range)
Creatinine: mean (range)
TNF-α and sTNF-R1 and sTNF–R2: mean ± s.d.
[a] $p < 0.001$ versus controls, [b] $p < 0.001$ versus ESRF; ANOVA
TNF-α and sTNF-R: measured by ELISA

Again, no difference in sTNF-R1 and -R2 levels between dialysis patients dialyzed with polysulphone versus hemophane membranes, nor could we detect a difference in sTNF-receptor plasma levels in the course of the dialysis procedure. Measurements of TNF-α and both sTNF-R at start, after 30 minutes and at the end of the dialysis procedure showed no significant increase (data not shown). All plasma concentrations of sTNF-R in patient groups were 15-30 times higher compared to healthy controls. In patients with ESRF plasma levels of sTNF-R1 and sTNF-R2 were lower compared to levels of sTNF-R1 and-R2 found in patients on renal replacement therapy, suggesting that some residual renal function is present. Positive correlation between sTNF-R1 and sTNF-R2 with serum creatinine has already been reported [Descamps-Latscha et al 1995]. We confirmed this correlation (sTNF-R1:r=0.85; sTNF-R2:r=0.75, $p<0.001$, Fig 3).

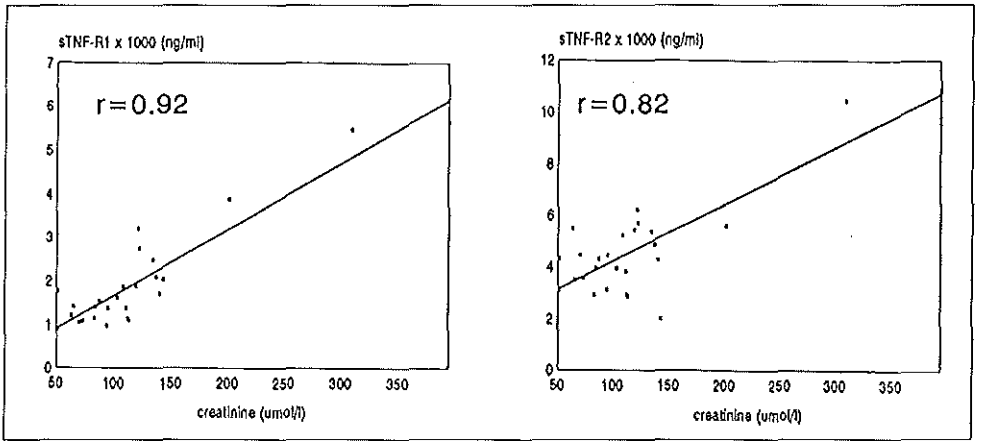

Figure 3:
Correlation between serum creatinine and sTNF-R1 (left panel) and sTNF-R2 (right panel) in patients with ESRF, on CAPD and on CIHD

Incubation of control plasma with recombinant TNF-α solution (final concentration=300 pg/ml) had no effect on the immunoreactive TNF-α-level, resulting in a recovery of 100% (range 92-104). In contrast, incubation of patients plasma resulted in a recovery of only 75% (CIHD), 72% (CAPD) and 90% (ESRF); $p < 0.05$: CAPD and CIHD versus controls (Fig 4). Thus, the high concentrations of TNF-α antagonists can act as a buffer for high, toxic concentrations of TNF-α, produced by activated PBMC of patients on chronic HD.

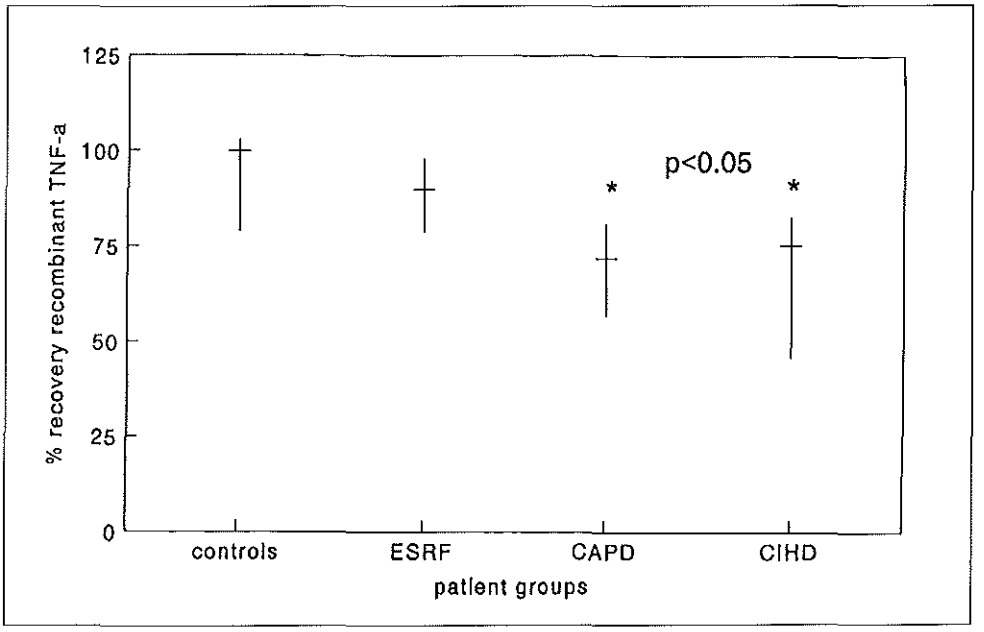

Figure 4:
Recovery recombinant TNF-α, after 30 minutes incubation, measured by ELISA in different patient groups.

Discussion

Many studies on the TNF-α system in HD patients have been reported during the last decade. Dinarello et al and Pereira et al are the founders of the cytokine paradigm in hemodialysis. Nevertheless, in spite of many publications only Pereira [1994] was able to detect TNF-α in unstimulated, peripheral blood, using a sensitive RIA method. Descamps-Latscha used ELISA technique and detected TNF-α in sera of ESRF, patients on CAPD and hemodialysis. She and her co-workers did not perform mRNA measurements, but determined other parameters of monocyte and T-cell activation, like neopterin and soluble CD25. Others were only capable of detecting TNF-α after stimulation with mitogens or LPS. In the literature it is reported that the various available TNF-α ELISA kits show a wide range in outcome [Kreuzer et al 1994]. These different ELISA kits give non-comparable results, due to interference of TNF-α binding proteins or circulating antagonists [Terlizzese et al 1996]. The ELISA kit, used

in our study, was not able to detect free TNF-α protein in patients with ESRF, on CAPD and CIHD. To overcome this technical complication we measured mRNA-expression for TNF-α by RT-PCR. Messenger RNA coding for TNF-α was present in all PBMC, including those of healthy controls. Significantly more TNF-α mRNA was detected in PBMC obtained from patients on CIHD versus all other groups ($p < 0.0001$). However, despite the high levels of mRNA-TNF-α, we were not able to detect significant levels of free TNF-α in peripheral blood. Besides, we found no increase in TNF-α concentrations during hemodialysis procedures. We did not find differences in concentrations of TNF-α or the soluble TNF-receptors between the two dialyzer membranes used, indicating that no difference in activation of the PBMC due to blood-membrane contact could be detected. We, therefore, considered the patients on CIHD as one group. In contrast, high levels of soluble TNF-receptors were present in pre-dialysis ESRF, and even higher soluble TNF-receptor levels were found in patients on renal replacement therapy, CAPD or CIHD, suggesting that renal function is requisite for clearance of the soluble receptors. This is supported by the linear correlation between the soluble receptors R1 and R2 and serum-creatinine with r-values of 0.85 (sTNF-R1) and 0.75 (sTNF-R2), $p<0.001$.

In spite of the low molecular weight, clearance of the soluble receptors in any form of dialysis, CAPD or CIHD, is very poor. As sTNF-receptors are produced after binding of TNF-α with the membrane receptors, the sTNF-receptors in peripheral blood reflect the presence of TNF-α and reveal the overall activity of the TNF-α system when renal clearance is not impaired. High levels of sTNF-receptors in patients with progressive renal failure and on renal replacement therapy are the resultant of production of TNF-α, together with the impaired renal clearance. The substantial difference between the patients on CIHD versus patients on CAPD in regard to the TNF-α mRNA indicates that the TNF-α system in CIHD is more activated. We assume that this activation is caused by repeated contact of mononuclear cells with the dialyzer membrane. This postulation is supported by our findings in PBMC of patients on CAPD. The levels of TNF-α mRNA in PBMC of patients on CAPD were in the range of healthy controls and ESRF patients, suggesting that no activation of the TNF-α system is present. Thus, monocyte activation by the blood-membrane interaction results in high levels of TNF-α mRNA expression by PBMC and high levels of sTNF-receptors in patients on chronic hemodialysis. Decreased recovery of TNF-α after incubation with CIHD and CAPD plasma reveals that the buffer capacity of the high levels of these sTNF-

receptors is evident, resulting in partial blockade of the cytotoxic and inflammatory actions of TNF-α. Blocking the inflammatory actions of TNF-α can be effective in treatment of acute rheumatoid arthritis by ameliorating the clinical symptoms [Baumgartner et al 1996]. However, an increased mortality is reported among patients in septic shock treated with anti-TNF-α antibodies. These unfavorable results are explained as possible toxic effects of the antibody, the deleterious effects of complete removing of TNF-α from the circulation or the prolonged presence of TNF-α in the circulation bound to the antibody, which acts as a buffer [Fischer et al 1996]. The cytokine paradigm makes TNF-α hold for the hemodialysis related morbidity, like hypotension, nausea, fever and amyloidosis. However, there is no clinical evidence that TNF-α alone is responsible for the HD related morbidity. Other cytokines, like IL-1β, IL-6 or IL-8, may be important mediators in this clinical situation as well. Unbalance between these cytokines and their antagonists may be of more importance than cytokine levels alone. The unbalance between TNF-α and its soluble receptors, found in our patients may contribute to the immunodeficiency and the higher susceptibility to infections and malignancies, due to the above mentioned reasons. A rational way to prevent this increased immunocompromised condition in these patients, is to increase the clearance of the soluble TNF-receptors and thus decrease the TNF-α buffercapacity to a level, which is seen in healthy controls. Successful kidney transplantation favors the renal clearance of the sTNF-receptors and may restore the unbalanced TNF-α system. However, in spite of a good renal function after renal transplantation, reaching creatinine clearance of 80 ml/min, the unbalance is only in part restored, leading to a persistent immunocompromised state [Van Riemsdijk et al 1998].

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CHAPTER 3

Quantitative flow cytometry shows activation of the TNF-α system but not of the the IL-2 system at the single cell level in renal replacement therapy (RRT)

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Nephrology Dialysis Transplantation (submitted)

Abstract:

Background: The immunological dysfunction in patients on hemodialysis may be related to imbalanced cytokine systems, like Tumor Necrosis Factor (TNF)-α and interleukin (IL)-2. Despite activation of these systems, hemodialysis patients show high susceptibility for infections, malignancies and have a poor immunological reaction on T-cell dependent antigens, like hepatitis B vaccination. In this study we have determined the activation status of the two different cytokine systems, at a single cell level, using the quantitative flow cytometry.

Methods: Using fluorescein-isothiocyanate (FITC) or phycoerythrin (PE) conjugated antibodies directed against the TNF-R2 (CD120b), IL-2Rα (CD25) and IL-2Rβ (CD122), we measured the expression of these receptors at a single cell level, in order to determine the level of activation of monocytes and T-lymphocytes.

Results: Significantly higher expression of the TNF-α receptor, TNF-R2, was present on both monocytes and T-lymphocytes in patients on renal replacement therapy (RRT) compared to pre-dialysis end-stage renal failure (ESRF) and controls, indicating activation of the TNF-α system. In contrast to the IL-2R expression, which was comparable in all patient groups and healthy controls, is suggestive for a non-activated state of the IL-2 system.

Conclusions: The present study illustrates the activated state of the TNF-α system in patients on renal replacement therapy, at a single cell level, while the IL-2 system seems not affected. These findings support the hypothesis that the interaction between the TNF-α and IL-2 cytokine systems is disturbed, leading to a increased susceptibility for infections and malignancy.

Key words: activation; ESRF; IL-2; quantitative flow cytometry; RRT; TNF-α

Introduction

Patients with end-stage renal failure (ESRF) and on renal replacement therapy (RRT) suffer from a high susceptibility for infections and show a higher incidence of malignancies compared to healthy controls [1]. This immune incompetence may be due to imbalanced defence mechanisms in which cytokines derived from antigen presenting cells (APC) and T-cells play a central role. Kimmel et al reported that the immunological dysfunction in patients on hemodialysis (HD) is related to the overall survival [2]. In patients with ESRF, on peritoneal dialysis (CAPD) or hemodialysis (HD) various cytokine systems are affected [3-5]. TNF-α is a pro-inflammatory cytokine, that induces expression of MHC class I molecules, activates the production

of enzymes and adhesion molecules. It can induce programmed cell-death and is needed for T-cell proliferation. It is mainly produced by activated monocytes and macrophages and, to a lesser extent by lymphocytes [6]. Therefore, it provides a pivotal role in the function of APC, whereas IL-2 is regarded as a central T-cell cytokine, that promotes expansion of T-cells, augments the cytolytic activity of NK cells, is involved in programmed cell death of activated T-cells and in the synthesis of immune globulins by B-cells [7]. Measurements of free plasma TNF-α appeared to be difficult. Variable results of TNF protein measurements by commercially available ELISA kits complicated the comparison between studies [8; 9]. In addition, active TNF-α is unstable and inadequate collection of blood samples may result in undetectable circulating cytokines as also the IL-2 protein measurements are influenced by mode of collection [10]. In contrast, measurements of the expression of membrane-bound activation markers by flow cytometry provide an elegant, reproducable and sensitive tool to determine the activated state of cytokine systems at the single cell level. In this study, we used flow cytometry to quantitate the expression of activation markers of both the TNF-α and IL-2 system: the TNF-R2 (CD120b) on lymphocytes and monocytes and the IL-2Rα chain (CD25) and the IL-2Rβ chain (CD122) on α/β T-cell Receptor (TCR) positive T-cells.

Patients and methods

Patients

In 11 patients (3 males, 8 females, mean age: 45.6 ± 19 years) with pre-dialysis ESRF, 8 patients (7 males, 1 female, mean age: 44.5 ± 14 years) on CAPD and in 12 patients (6 males, 6 females, mean age: 57.8 ± 15 years) on HD the activation markers of the TNF-α and IL-2 system were determined on peripheral T-lymphocytes and monocytes, using quantitative flow cytometry. Mean serum creatinine was 552 μmol/l (ESRF), 1031 μmol/l (CAPD) and 995 μmol/l (HD). The patients with pre-dialysis ESRF had a mean creatinine clearance of 13 ± 2.5 ml/min. In HD patients a Kt/V of 1.3 was obtained, while patients on CAPD were dialyzed and obtained a Kt/V of 1.5. Mean time on dialysis was for CAPD patients 27.4 ± 8.3 months and for HD patients 26.1 ± 5.5 months. The dialyzer membranes used in the study were Polysulphone (F60 Fresenius, AG, Bad Homburg, Germany) and Hemophane (MA-12H, Kawasumi Laboratory Inc., Minamiohi, Tokyo, Japan). Both dialyzers are known for their better bio-compatibility, compared to cuprophane membranes [11]. Previously we have shown that patients dialyzed with either of the two membranes showed no differences

in activation parameters of the TNF-α system [12]. The HD patients were on bicarbonate dialysate. The dialysate is routinely cultured and no periods of contamination were found (culture results: < 10^3 micro organisms per ml.). The patients on CAPD used Baxter twinbag system 2 liter/four times daily. Before, during and at least 4 weeks after blood collection the patients on CAPD had no signs of peritonitis. Blood samples were collected during infection-free period. In HD patients blood samples were collected before start of the hemodialysis procedure. At time of blood sample collection no patients were known to have a malignancy. In ESRF and CAPD patients blood samples were collected during routinely outpatient control visits. Causes of renal failure were hypertension (n=10), membranous glomerulonephritis (n=8), Wegener's granulomatosis (n=2), focal segmental glomerulosclerosis (n=2), polycystic kidney disease (n=4), IgA nephropathy (n=1), amyloidosis (n=1), unknown (n=3). Most patients used anti-hypertensive drugs: calcium-entry blockers, ACE-inhibitors, β-blockers. Subcutaneous recombinant erytropoietine treatment is installed in all patients with hemoglobin levels lower than 6 mmol/l (=9.7 g/dl). None of the patients used corticosteroids. Amongst the patients and controls there were no diabetics. All data were compared to those of 9 healthy controls, who were not on medication (4 males, 5 females, mean age: 35.6 years, mean creatinine < 100 μmol/l).

Methods

Sample preparation and flow cytometric analysis

Blood samples were collected in pyrogen-free tubes, containing EDTA in a final concentration of 1 mg/ml. Whole blood EDTA samples were monitored for the presence of the immune competent cells: monocytes (CD14+) and lymphocytes (α/β TCR positive). Surface activation markers were analyzed by two-colour flow cytometry after staining with monoclonal antibodies directed against CD14 (Immunotech, Marseille, France) as marker for the monocytes, and WT31 (Becton Dickinson, Mountain View, CA, USA) as a marker for the α/β chain of the T cell receptor (TCR). In these subsets CD25 (IL-2Rα, Becton Dickinson), CD122 (IL-2Rβ, Becton Dickinson) and CD120b (TNF-R2, Immunotech) were monitored. The antibodies, except CD120b, were directly conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE). For CD120b we used a two-step staining. After the first step with CD120b, cells were incubated with $F(ab)_2$ Goat-anti-Rat IgG PE. The staining procedure was performed by incubating 15 μl 1/100 diluted CD120b antibody with 100 μl blood (30 min, at 4 ^{0}C). After washing in Hanks Balanced Salt Solution

(HBSS, Gibco BRL, Paisly, UK) with 0.1% Bovine Serum Albumin (BSA, Sigma, St Louis, MO, USA) and 0.01% sodium azide (Merck, Darmstadt, Germany), the red blood cells were lysed by FACS Lysing Solution (Becton Dickinson). Samples were centrifugated and washed in Cell Pack (TOA, Hamburg, Germany). Flow cytometric analysis was performed on FACscan flow cytometer using Cell Quest software (Becton Dickinson). From each tube 10000 events (α/β TCR positive, CD14+) in the gate were measured. In order to compare the measurements in time the flowcytometer was calibrated using specific calibration beads (Calibration Beads Quantum 1000, Flow cytometry Standards Corp. San Jose, PR, USA). Each bead contains a known amount of fluorochrome. The intensity of the fluorescence is converted to a standard curve using Quick Cal program for Quantum Beads (Becton Dickinson). The mean fluorescence is denoted as molecular equivalents of fluorochrome, MESF. Figure1 .

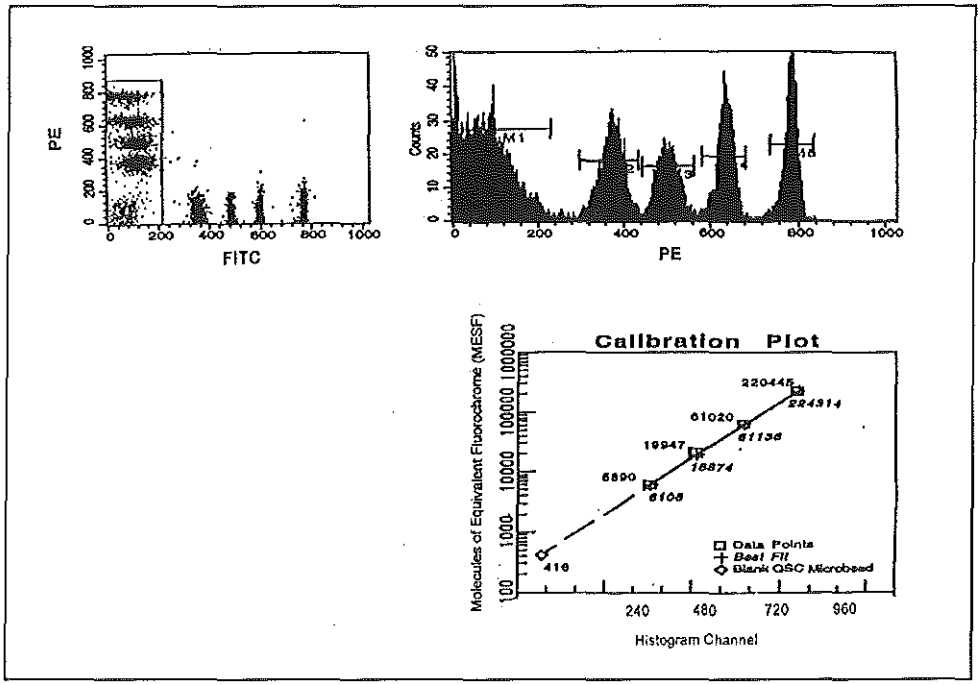

Figure 1:
Standard curve fluorescence intensity of calibration beads

Upper panel: Flowcytometric analysis of calibration beads, each of which contains a specific amount of fluorescine.

Lower panel: Standard curve of fluorescence intensity constructed after flowcytometric analysis of calibration beads, using Quick Cal program for quantum beads software. The intensity of the fluorescence is depicted as molecular equivalents of soluble fluorochrome (MESF)

Relation between serum creatinine and membrane receptor expression was analyzed for the whole group (patients and controls, n=40), using the Spearman Rank Correlation. Correlation between time on dialysis (CAPD and HD) and membrane receptor expression was also evaluated by the Spearman Rank Correlation test.

Statistics

Data are recorded as mean ± SEM, or median and range. Absolute numbers of lymphocytes and monocytes in the patient groups were compared with the unpaired student's t test. Differences in receptor expression between patient groups were analyzed using one-way ANOVA test, while differences between groups, separately, were analyzed using Mann Whitney test. The Spearman r correlation coefficients were used to determine the relationship between serum creatinine, time on dialysis and the membrane receptor expression (IL-2Rα (CD25), IL-2Rβ (CD122), TNF-R2 (CD120b) on monocytes and lymphocytes). P-values ≤0.05 were considered as significant.

Results

Absolute number of α/β TCR positive T-cells were significantly lower in HD patients than in healthy controls: 665±88 cells/μl versus 979±99 cells/μl, p=0.02. In patients on CAPD (673±116 cells/μl) and ESRF (738±172 cells/μl) the absolute number of α/β TCR positive T-cells were lower than in the control group, p=0.07 and p=0.22, respectively, although not statistically significant. The absolute number of monocytes was comparable between all groups: 314±34 cells/μl (controls), 284±29 cells/μl (ESRF), 374±59 cells/μl (CAPD) and 293±56 cells/μl (HD).

Expression of the activation markers of the IL-2 system (i.e. CD25, CD122) was in the same range for patients and controls. The mean expression of the IL-2Rα (CD25) varied from 779±132 MESF (controls) to 992±163 MESF (CAPD), 762±49 MESF (ESRF) and 763±104 MESF (HD), p=0.51, ANOVA. The mean expression of the IL-2Rβ (CD122) ranged from 365±14 MESF (controls), 409±19 MESF (ESRF), 401±18 MESF (CAPD) and 431±38 MESF (HD), p=0.40, ANOVA. Figure 2.

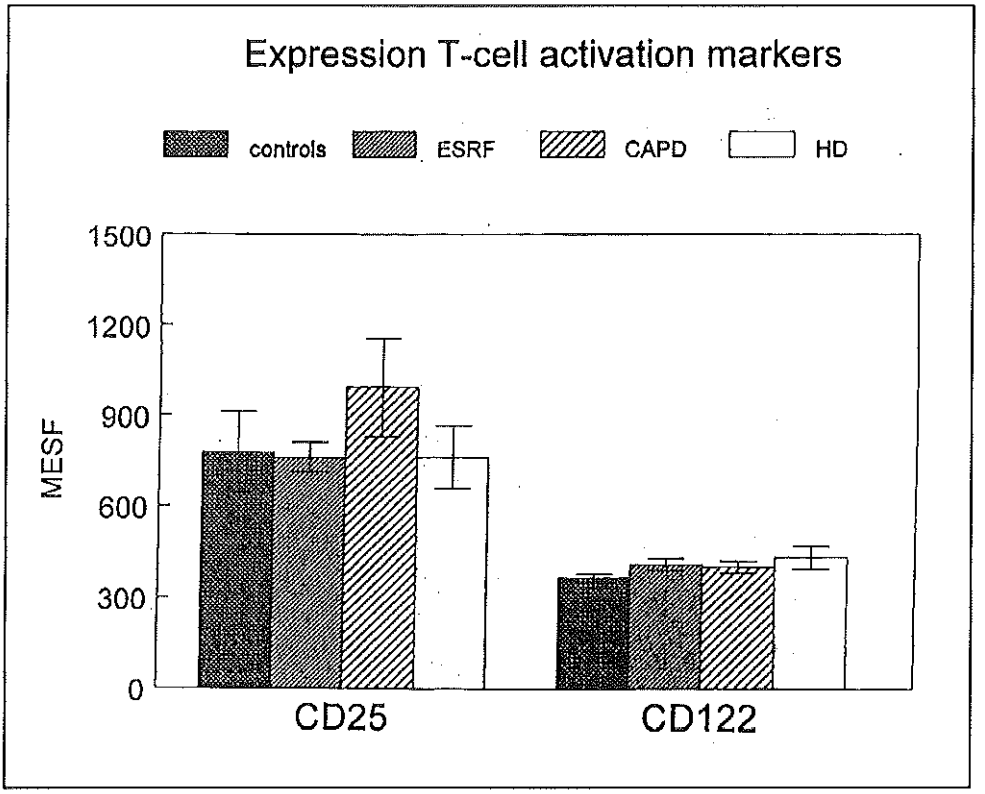

Figure 2:
Expression of T-cell activation markers: CD25 (IL-2rα chain) and CD 122 (IL-2Rβ chain) in patients with end-stage renal failure (ESRF), on peritoneal dialysis (CAPD) and hemodialysis (HD) measured with quantitative flow cytometry in peripheral blood. The quantity of fluorescence is depicted as molecular equivalents of soluble fluorochrome (MESF). Tat error bars are SEM.

In contrast to these comparable expression levels of the activation markers of the IL-2 system on T-cells, the expression levels of the activation marker of the TNF-α system, TNF-R2 (CD120b) were significantly higher in patients on renal replacement therapy, both on monocytes and on α/β TCR positive lymphocytes, p=0.02 and p=0.03, respectively (ANOVA). The mean TNF-R2 (CD120b) expression on lymphocytes was

3153±508 MESF (controls) versus 3451±288 MESF (ESRF), p=0.65, in CAPD: 5670±997 MESF, p=0.06, and in HD patients the expression: 5466±893 MESF, p=0.01. Mean TNF-R2 (CD120b) expression on monocytes was also higher in patients on CAPD and HD, but not in ESRF compared to controls. MESF (CAPD): 7285±1516 versus 2564±808 (controls), p=0.005. MESF (HD): 10233±3531, p=0.02, Mann-Whitney Test. Figure 3.

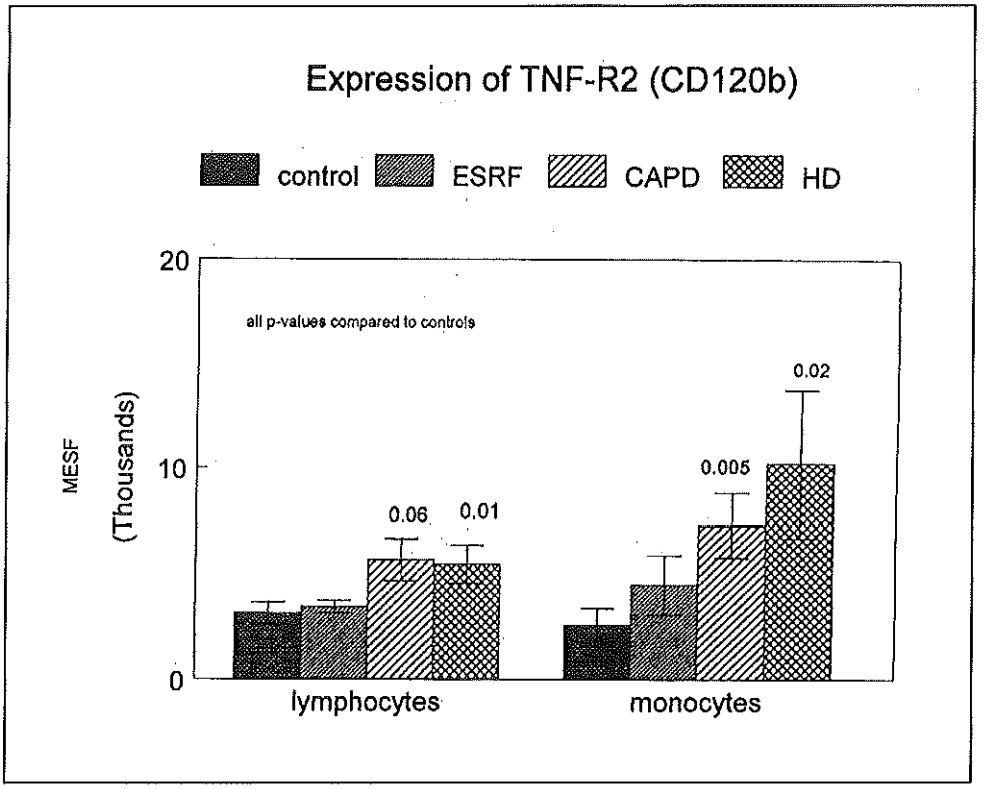

Figure 3:
Expression of the TNF-α activation marker: CD 120b (TNF-R2) on lymphocytes and monocytes in patients with end-stage renal failure (ESRF), on peritoneal dialysis (CAPD) and hemodialysis (HD) compared to healthy controls, measured by quantitative flow cytometry. The quantity of fluorescence is depicted as molecular equivalents of soluble fluorochrome (MESF). Tat error bars are SEM.

Of the studied cytokine activation markers (CD25, CD122, CD120b on lymphocytes and monocytes) only CD120b on monocytes showed a correlation with serum creatinine. R-coefficient for CD25 was 0.08 (p=0.62), for CD122: r=0.16 (p=0.31), CD120b on lymphocytes: r=0.16 (p=0.31), and CD120b on monocytes: r=0.38 (p=0.03), Spearman Rank Correlation. Except for CD120b expression on monocytes we found no correlation between age and cytokine receptor expression: CD25: r=-0.16 (p=0.32), CD122: r=0.13 (p=0.42), CD120b on lymphocytes: r=0.24 (p=0.16), and CD120b on monocytes: r=0.43 (p=0.01), Spearman Rank Correlation. Also time on dialysis was not correlated with the expression of the membrane receptors: r=0.12, p=0.68, Spearman Rank Correlation.

Discussion

In the present study, we used quantitative flow cytometry to evaluate the expression of activation markers of the TNF-α and the IL-2 system in patients with ESRF and on renal replacement therapy. We determined the receptor expression for TNF-α and IL-2 on immune competent cells, α/β TCR positive lymphocytes and the CD14+ macrophages in order to differentiate which cells, T-cell or APC, are activated. To evaluate the activation of the IL-2 system we measured the expression of the IL-2Rα (CD25) and the IL-2Rβ (CD122). In this study we were predominantly interested in the expression of the TNF-R2, because TNF-α action directed by TNF-R2 results in proliferation of T-cells. We found reduced absolute numbers of lymphocytes in the presence of comparable absolute numbers of monocytes in patients on HD versus all other groups. It is known that activated lymphocytes are sequestered in the capillaries of the lung after blood dialyzer membrane contact [13]. Deenitchina et al reported that this lymphopenia was the consequence of low numbers of CD4+ T-cells [14]. We found an increased expression of the TNF-R2 on both monocytes and lymphocytes from patients on renal replacement therapy, which is suggestive for activation of the TNF-α system [15]. This coincides with the high levels of soluble receptors and the significantly higher levels of free plasma TNF-α in these patients [16]. In contrast, in patients with ESRF TNF-R2 expression was comparable to the expression measured on immune competent cells from healthy controls. This shows that the TNF-α system is not activated in ESRF patients. As the creatinine clearance in the patients with ESRF in this study was 13 ml/min, which is comparable to the creatinine clearance obtained in patients on renal replacement therapy, i.e. CAPD or HD, renal insufficiency alone, can not be responsible for the differences in expression of the TNF-α activation marker,

TNF-R2, found in these patients. Therefore, we think that the increased TNF-R2 expression is not caused by renal insufficiency itself, but by renal replacement therapy, i.e. CAPD or HD. The expression of TNF-R2 on lymphocytes and monocytes is comparable between patients on CAPD and HD. The mode of renal replacement therapy is apparently not important for this TNF-R2 expression on lymphocytes or monocytes. After TNF-α binding to its membrane receptor, the extracellular domains are split off and can be identified as soluble TNF-R in peripheral blood. Other causes of shedding the membrane receptor are poorly understood. The sTNF-R are predominantly metabolized by renal clearance. Due to impaired renal clearance levels of sTNF-R show a strong positive correlation with serum creatinine and renal function [12; 17]. In the present study we found no correlation between the expression of CD25, CD122 and CD120b on lymphocytes and monocytes and serum creatinine per patient group. Nor did we found a correlation between time on dialysis (CAPD or HD) and the expression of the membrane receptors. The expression of the TNF-R2 (CD120b) on monocytes, seemed positively correlated with progressive renal insufficiency when the test was performed with all subjects, but not in the separate patient groups. Age may be another confounding factor in this study. In literature conflicting data are reported concerning cytokine levels and production in aged subjects. Higher [18-22] as well as lower [20; 23; 24] levels and production capacity of IL-2 and TNF-α in aged subjects are described. In the present study the mean age between the patient groups and controls is significantly different. However, the difference in mean age between the patient groups among themselves (ESRF versus CAPD and HD) is not significant, thereby showing that the increased expression of the TNF-R2 on cells of ESRF, CAPD and HD patients is the result of their renal insufficiency. Various drugs are known to be of influence on TNF-α levels. Recombinant erytropoietine and Angiotensin-Converting-Enzyme (ACE-) inhibitors may induce TNF-α [25; 26]. In this study all patients are on various medication. No differences in the use of ACE-i or erytropoietine between the various patient groups is present, and therefore the influence of the medication is not seen as a dominant factor influencing the results of the TNF-R2 expression.

Despite the high expression of TNF-R2 on lymphocytes the T-cells seem not activated as shown by normal expression of the IL-2 activation markers on the cell membrane. The interaction between the TNF-α system and the T-cells appears to be inadequate, which may result in the high incidence of infection and malignancy found in HD patients. Previously, we found that the Phytohemagglutinin (PHA-) stimulated TNF-α

production by PBMC was significantly reduced in patients on HD, while PHA-stimulated IL-2 production was comparable to the production by PBMC obtained from healthy controls [16]. Others reported differences between CAPD and HD patients in the production of specific T-cell cytokines. In CAPD the mitogen phorbol-12-myrisate-13acetate (PMA-) stimulated T-cell production of TNF-α and IL-2 was lower compared to the production by T-cells obtained from HD patients [27]. We suggest this impaired TNF-α production results from "exhaustion" due to continuous activation of the TNF-α system. In HD patients this is explained by chronic blood-membrane contact, in CAPD patients by chronic, low-grade, subclinical intra-peritoneal inflammation [28-30]. TNF-α activity, conducted by TNF-R1 is responsible for signaling apoptosis, while TNF-α activity conducted by TNF-R2 is responsible for signaling proliferation of thymocytes and cytotoxic T-cells [15]. A distinct difference in action is, however, not overt. Monoclonal antibodies directed against TNF-R2 can partially antagonize the same TNF responses that are induced by TNF-R1. Tartaglia et al reported that specific activation of TNF-R2, in the presence of PHA, results in stimulation of human T-cells [30]. The increased expression of the TNF-R2 may thus result in T-cell activation and proliferation in the presence of TNF-α and mitogen. In summary, we found an activated TNF-α system shown by high expression of the TNF-R2 on lymphocytes and monocytes. In previous studies we already found increased mRNA expression for TNF-α and elevated plasma TNF-α protein levels [12]. The IL-2 system seems not activated, as shown by the comparable expression of the T-cell activation markers, IL-2Rα (CD25) and IL-2Rβ (CD122) in patients and controls. The immunoregulatory activity of TNF-α, which results in induction of the IL-2R expression and enhances T-cell responses directed by IL-2 [31], failed to induce T-cell activation in our studied patient groups.

The present study shows that quantitative flow cytometry provides additional information about the activated state of cytokine systems at a single cell level, and clarifies the nature of the immune competent cells involved in the immune responses in patients with ESRF and on renal replacement therapy. Our results show that interaction between APC (TNF-α) and T-cells (IL-2) is disturbed, which may contribute to the immune deficiency found in those patients.

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CHAPTER 4

Tachyphylaxis for TNF-α production by T-cells resulting from activation of the TNF-α system during hemodialysis

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Abstract:

Background: The immunosuppressive state of hemodialysis patients is accompanied by activation of antigen presenting cell-derived cytokines, e.g. TNF-α, which are required for T-cell activation. To test whether an activated TNF-α system results in impaired T-cell response in these patients, we analyzed parameters of their APC function (e.g. TNF-α system) and T-cell function (e.g. IL-2 system).

Methods: By quantitative flow cytometry the expression of the TNF-receptor2 (TNF-R2 = CD120b) and the α- and β-chain of the IL-2R (CD25, CD122) were measured. Using RT-PCR the mRNA for TNF-α, IL-2 and IL-2R were determined. PHA- and IL-2 stimulated proliferation and cytokine production were measured. Biological activity of soluble receptors was measured by adding recombinant cytokines to patient's plasma.

Results: CD120b expression was significantly increased in HD patients, whereas CD25 and CD122 was comparable to controls. In contrast to mRNA for IL-2 and IL-2R, mRNA for TNF-α was increased in HD. This resulted in significantly increased TNF-α levels in HD patients. In peripheral blood of HD patients high levels of soluble TNF-R (R1 and R2) and IL-2R were found. These receptors were capable of binding 40% of added TNF-α and 55% of added IL-2. PHA-induced TNF-α production by T-cells from HD patients was significantly lower, while their PHA stimulated IL-2 production and proliferation capacity by T-cells were comparable to controls.

Conclusions: We conclude that although the TNF-α system is activated during hemodialysis the TNF-α production of T-cells is impaired, suggesting tachyphylaxis of T-cells for TNF-α as their proliferation capacity and IL-2 production capacity do not imply an intrinsic T-cell defect.

Key words: tachyphylaxis, TNF-α, T-cells, hemodialysis

Introduction

Patients on chronic hemodialysis suffer from general immunosuppression, leading to a high susceptibility for infection and cancer and show a deficient response on T-cell dependent antigens, like hepatitis B and influenza vaccination (1;2).

This depression of T-cell activity might be the result of a defect in their antigen presenting cell (APC) function, in which the pro-inflammatory cytokine TNF-α plays a prominent role. However, the immunusuppressive state of HD patients seems to be accompanied by activation rather than by impairment of the monocyte/macrophage system, resulting in an increased production of cytokines such as TNF-α, IL-1β, IL-6

(3-6). Therefore, other factors than the TNF-α system might be involved in immune compromised state of HD patients, e.g. an intrinsic defect of T-cells to produce and to respond to IL-2. IL-2 promotes expansion of T-cells, augments the cytolytic activity of NK-cells, is involved in the synthesis of immunoglobulins by B-cells and protects activated T-cells from apoptosis (7-9). Immune reactivity, *in vivo*, however, is not only determined by cytokine production, but also depends on the presence and activity of their antagonists, thus on the bioavailibility of cytokines. Biological activity of cytokines is regulated by membrane bound receptors. For TNF-α, 2 receptors have been identified, R1 (p55) and R2 (p75), while IL-2 has its own IL-2R, consisting of three separate chains, IL-2Rα (CD25), IL-2Rβ (CD122), and IL-2Rγ (CD132) chain. After binding, the extracellular domain of the receptor splits off and can be identified as soluble receptors: sTNF-R1, sTNF-R2 and sIL-2R (9;10). These soluble receptors are mainly metabolized by renal clearance. However, their molecular weight prevents clearance by the dialyzer membrane. Recently, we found high levels of sTNF-R1 and sTNF-R2 in peripheral blood of patients on renal replacement therapy (11). These high levels appeared to be biological active and were able to buffer free, active TNF-α for 40%. Previously, IL-2 buffer capacity by sIL-2R (sCD25) was also described (10). Thus, the immunosuppressive state of HD patients may be due to a deficient cytokine production capacity, an intrinsic T-cell defect, or a disturbed APC/T-cell interaction as result of biological active soluble cytokine receptors or deficiency in the costimulatory pathways. Data on T-cell function in HD patients are conflicting. Some authors have mentioned defective T-cell function, while others have reported normal T-cell function or even activation, depending of the dialyzer membrane used (7;8;12-14). Previously, we already demonstrated no differences in mRNA expression for IL-2 in patients with pre-dialysis end-stage renal failure (ESRF), on peritoneal dialyisis (CAPD) or hemodialysis (HD) or in the expression of T-cell activation markers (CD25, CD122, HLA-DR) (15). To clarify the contribution of the cytokines, TNF-α and IL-2, on the immune compromised state of HD patients, we evaluated both cytokine systems in more detail. We measured cytokine plasma levels, mRNA expression (RT-PCR), cytokine production capacity of PBMC (ELISA), TNF-receptor (CD120b = TNF-R2) expression on monocytes and lymphocytes, and IL-2Rα, (CD25), IL-2Rβ (CD122), and HLA-DR expression on α/β positive T-cells. We performed IL-2 recovery studies to evaluate the biological activity of the sIL-2R (sCD25). In addition, we analyzed PHA- and IL-2 induced T-cell proliferation in own uremic plasma, and in pooled healthy

serum, to determine the influence of the uremic environment on proliferation capacity of healthy and HD peripheral blood mononuclear cells (PBMC).

Patients, Materials and Methods

Patients

15 patients on hemodialysis (HD, 8 males, mean age: 63 ± 15 yrs, serum creatinine: 985 ± 169 μmol/l) were evaluated. The patients were dialyzed 2-3 times/week, 4-6 hours (645 ± 112 minutes/week). A mean Kt/V of 1.3 was obtained in all patients. Time on dialysis varied from 8 till 91 months, mean 30 months. The dialyzer membranes used in the study were Polysulphone (F60 Fresenius, AG, Bad Homburg, Germany, n=9) and Hemophane (MA-12H, Kawasumi Laboratory Inc., Minamiohi Shinagawa, Tokyo, Japan, n=6). Both dialyzer membranes belong to the 'high-flux' membranes and are known for their bio-compatibility. Previously, we have shown that no differences in the activation parameters of the TNF-α system were present between patients dialyzed with either of the membranes (11). All patients were on bicarbonate dialysate. The dialysate was routinely cultured, there were no periods of contamination, culture results were $< 10^3$ micro-organisms per ml. All patients were on subcutaneously erytropoeitin, depending on their hemoglobin levels. The controls were healthy laboratory personel (n=11; 5 males, mean age: 35.6 ± 9.9 yrs, serum creatinine < 100μmol/l). Non-detectable data are counted as zero.

Sample preparation

Blood samples were collected in pyrogen-free tubes containing EDTA in a final concentration of 1 mg/ml at the start of the hemodialysis-procedure. Samples for flow cytometric analysis were processed immediately after collection. For the isolation of peripheral blood mononuclear cells (PBMC) the buffy coat was diluted in phosphate-buffered saline (PBS) solution and layered over a Ficoll-Isopaque gradient (δ = 1.077). Immediately following procurement, 2×10^6 cells were snap-frozen in liquid nitrogen and stored at -80 °C for RT-PCR analysis. Plasma was stored at -80 °C and the PBMC for *in vitro* assays were kept in liquid nitrogen.

Messenger RNA (mRNA) isolation and copy DNA (cDNA) reaction and Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

In PBMC we measured mRNA expression levels of TNF-α, IL-2 and IL-2R (CD25). Procedures for isolation of mRNA and reverse transcription into cDNA and RT-PCR were performed as previously described in detail (16). In brief, total RNA was extracted from snap-frozen PBMC (2.10^6) and subsequently cDNA was synthesized with random primers. Aliquots cDNA were directly used for PCR amplification using sequence-specific primers for TNF-α, IL-2, IL-2Rα (CD25) and the housekeeping gene keratin (16).

Quantification of mRNA expression by competitive template RT-PCR

To estimate the relative initial amount of functional TNF-α and IL-2R (CD25) mRNA in PBMC, a competitive template RT-PCR assay was used and comparison was made against the housekeeping gene keratin. The latter gene is assumed to be expressed at a constant level in PBMC. To obtain a standard curve for TNF-α, IL-2R (CD25) and keratin, known amounts of internal control fragment were added in different dilutions to constant amounts of sample cDNA for competitive co-amplification. The internal control was designed to generate a PCR product of a smaller size to allow differentiation between the amplified target and the internal control. The relative intensity of internal control and target products on gel was measured by luminescence with a DC-40 camera in combination with analysis software (Kodak, Rochester NY, USA). Subsequently, the relative concentrations of TNF-α and IL-2R (CD25) were divided by the relative concentration of keratin to estimate the initial cytokine mRNA expression level in PBMC. This represents the amount of TNF-α and IL-2R (CD25) mRNA transcripts corrected for the amount of mRNA used for reverse transcription and efficacy of each reaction.

Flow cytometric analysis

Whole blood EDTA samples were monitored for the presence of immune competent cells: monocytes (CD14+) and lymphocytes (α/β TCR positive). Surface activation markers were analyzed by two-color flow cytometry after staining with monoclonal antibodies directed to CD14 (Immunotech, Marseille, France), as marker for monocytes, and WT31 (Becton Dickinson, Mountain View, CA, USA) as a marker for the α/β chain of the T-cell receptor (TCR). In these subsets IL-2Rα (CD25), IL-2Rβ (CD122) and HLA-DR (Becton Dickinson) and TNF-R2 (CD120b, Immunotech) were

monitored. The antibodies, except CD120b, were directly conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE). For CD120b a two-step staining was used. After the first step with CD120b, cells were incubated with $F(ab)_2$ Goat-anti-Rat IgG PE. The staining procedure was performed by incubating 15 µl 1/100 diluted CD120b antibody with 100 µl blood (30 min, at 4 °C). After washing in Hanks Balanced Salt Solution (HBSS, Gibco, BRL, Paisly, UK) with 0.1% Bovine Serum Albumin (BSA, Sigma, St Louis, MO, USA) and 0.01% sodium azide (Merck, Darmstadt, Germany), the red blood cells were lysed by FACS Lysing Solution (Becton Dickinson). Samples were centrifugated and washed in Cell Pack (TOA, Hamburg, Germany). Flow cytometric analysis was performed on a FACscan flow cytometer using Cell Quest software (Becton Dickinson). From each tube 10000 events in the gate were measured. In order to compare different flow cytometric assays in time we calibrated the flow cytometer using specific Calibration Beads (Calibration Beads Quantum 1000, Flow cytometer Standards Corp, San Jose, PR, USA). Each bead contains a known amount of fluorochrome. The intensity of the fluorescence is converted to a standard curve by Quick Cal Program for Quantum Beads (Becton Dickinson). The mean fluorescence is denoted as molecular equivalents of soluble fluorochrome (MESF, Figure 1A+1B).

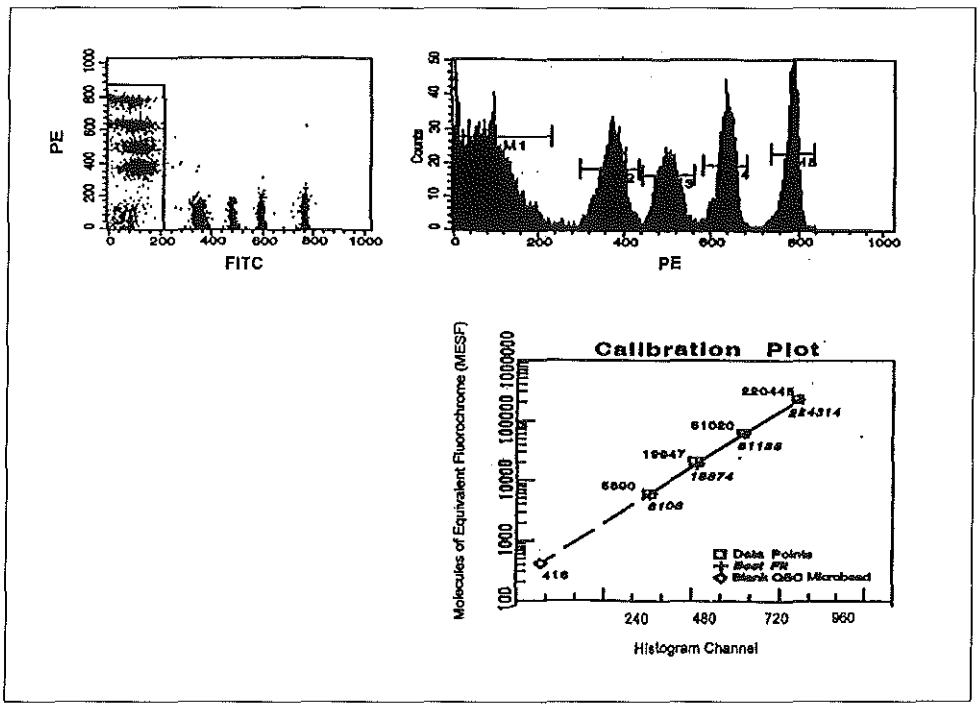

Figure 1A:

Quantitative flow cytometry:

Construction of the standard curve using specific calibration beads, each containing a different, known amount of fluorochrome (upper panel). The amount of fluorochrome is converted by software into fluorescence intensity, denoted as molecular equivalents of fluorochrome (MESF) (lower panel).

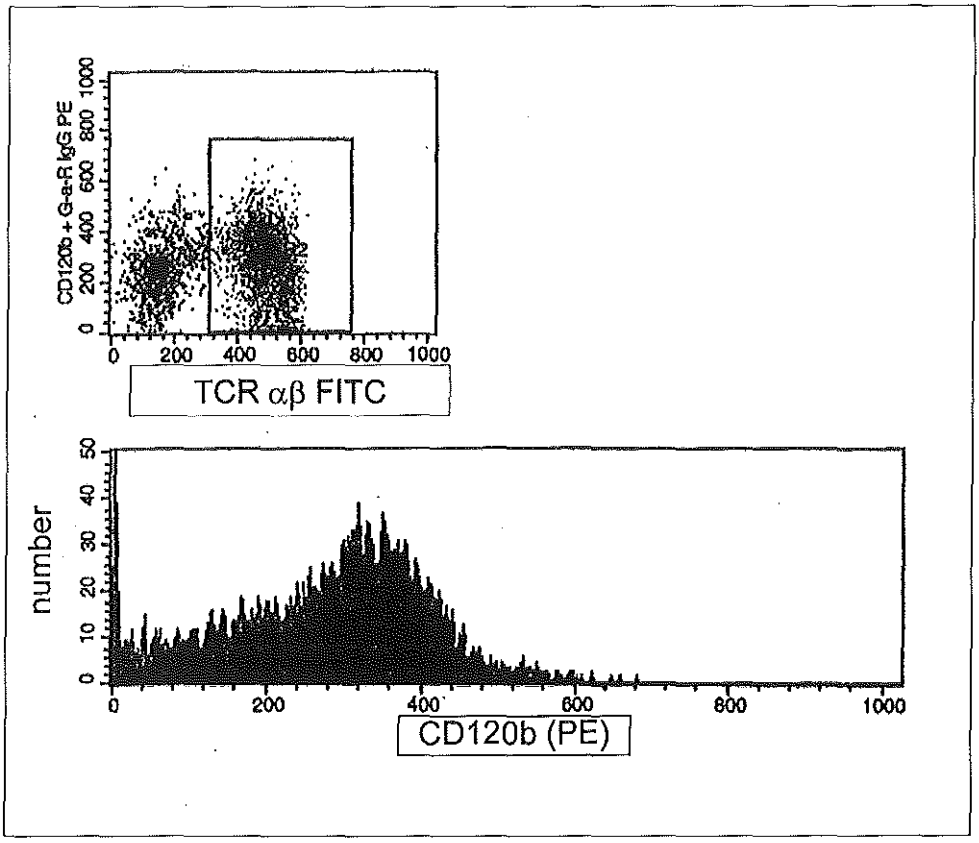

Figure 1B:
Typical example of CD120b expression on α/β TCR positive T-cells by quantitative flow cytometry. In the upper panel: the FITC labeled α/β positive T-cells are gated. In the lower panel the fluorescence intensity of the CD120b positive T-cells is depicted. The mean fluorescence intensity can be extrapolated to the standard curve and denoted as MESF (fig. 1A).

Enzyme Linked Immuno Sorbent Assay (ELISA)

TNF-α, IL-2 and IL-2R (CD25) concentrations were measured by commercially available enzyme-linked immunosorbent assays (ELISA, BioSource International, Inc., Camarillo, CA and Immunotech, Diagnostic Product Corporation, Los Angeles, CA, respectively). The detection limit for TNF-α is <0.09 pg/ml and the coëfficient of variation is 3.2-7.0%. The detection limit for IL-2 is 5 pg/ml, coefficient of variation 5-10%. The detection limit for IL-2R is 16 U/ml with a coefficient of variation of 5%.

Soluble TNF-receptors R1 and R2 were measured by ELISA (R&D Systems Europe, Abingdon, United Kingdom). The detection limit is 15 pg/ml for both sTNF-R1 and -R2. To evaluate the biological activity of the sIL-2R in HD, we performed a IL-2 recovery assay. The test was performed with 2 concentrations recombinant human (rh) IL-2: 125 pg/ml and 500 pg/ml. After 30 minutes incubation of patients and control plasma with a known amount of rh IL-2, the residual IL-2 level was measured using the above mentioned ELISA. The recovery is:

$$\text{Recovery} = \frac{[\text{measured IL-2}] - [\text{baseline} + \text{added rhIL-2}]}{\text{added rhIL-2}} \times 100\%$$

In vitro stimulation assays

To test the proliferation response of PBMC from HD patients and controls, we stimulated PBMC, *in vitro*, with phytohemagglutinin M (PHA-M), (1:100, final dilution, Difco Laboratories, Detroit, MI, USA) and with rhIL-2 (10 ng/ml, Chiron, Amsterdam, The Netherlands), for 3 days at 37 ^{0}C in a humidified atmosphere containing 5% CO_2. Proliferation after ^{3}H-thymidine (0.5μCi/well) incubation for 8 hours of culture, was measured. Radioactivity was determined using a Betaplate counter (LKB, Bromma, Sweden) and expressed as mean counts per minute (CPM). All assays were performed in own plasma and in pooled serum. Both plasma and pooled serum is heat-inactivated. The pooled sera are obtained from healthy blood donors. Each individual serum, included in the pool, is tested for the presence of HLA-specific antibodies and in a MLC using three different responder-stimulator combinations. The serum is excluded from the pool if antibodies or inhibitory, i.e. stimulatory effects on proliferation are present. To measure the influence of uremic environment on the proliferation capacity, PBMC obtained from healthy controls were stimulated with PHA or rhIL-2 in pooled healthy and uremic serum derived from HD patients. In the culture supernatants we analyzed the concentration of TNF-α, sTNF-R1 and R2, using the previously mentioned ELISA.

Statistics

The results are given as mean ± SD or SEM, or median with range, whatever was appropriate. Comparison of normally distributed variables was addressed by analysis of variance (ANOVA). Qualitative RT-PCR (IL-2 mRNA) results were analyzed by the Fischer Exact test. Quantitative RT-PCR data (IL-2R mRNA,TNF-α mRNA) and concentrations of cytokines in peripheral blood were compared by Mann Whitney U test. P values ≤ 0.05 were considered significant.

Results

Messenger RNA expression of TNF-α and IL-2 by RT-PCR

In PBMC obtained from patients on HD and healthy controls we measured the mRNA expression for TNF-α, IL-2 and IL-2R (CD25). TNF-α mRNA expression levels were significantly higher in HD patients than in healthy controls: median TNF/keratin ratio 47541 versus 604, $p<0.0001$ (Figure 2). In contrast, IL-2 mRNA expression was less often found in PBMC from HD patients compared to healthy controls, 6 out of 15 (38%) in HD versus 9 out of 11 (88%) in controls, $p=0.34$. These results are in line with our previous study, in which we could not detect mRNA expression for IL-2 in patients with ESRF, on CAPD or on HD, neither in healthy controls (17). The mRNA expression levels of the IL-2Rα-chain (CD25) was also not different in patients and healthy controls: mean IL-2R/keratin ratio: 22.9 versus 14.2, n.s. (Figure 2).

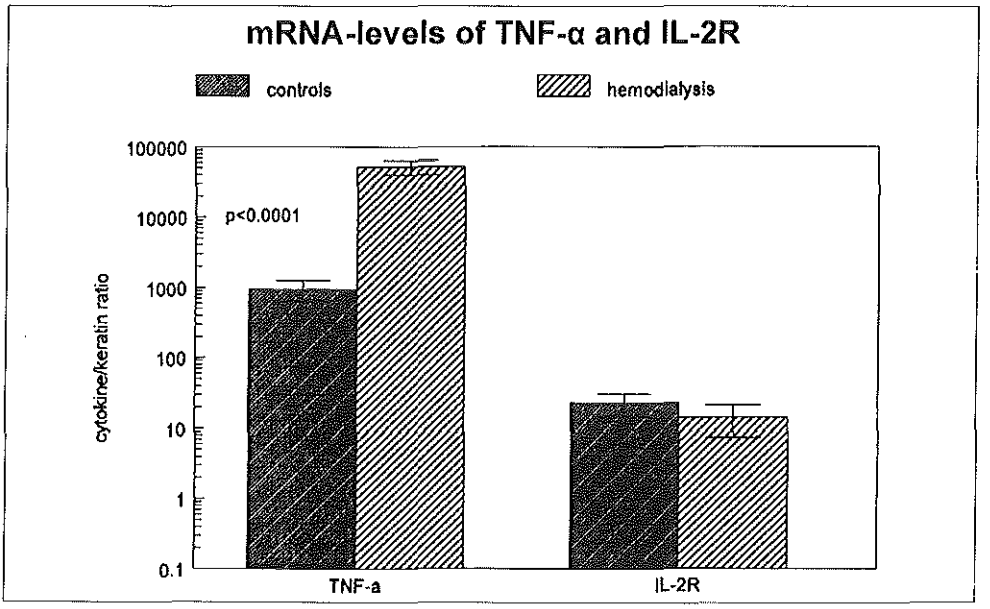

Figure 2:
Messenger RNA expression of TNF-α and IL-2R by peripheral blood mononuclear cells (PBMC) in patients on hemodialysis and healthy controls measured by RT-PCR. Results are denoted as cytokine/keratin ratio, in which keratin is the house-keeping gene.

TNF-α and IL-2 receptor expression on immunocompetent cells by quantitative flow cytometry

The absolute numbers of α/β positive T-cells were significantly lower in HD patients compared to healthy controls: mean 664 ± 87/μl versus 979 ± 99/μl (p=0.03). The absolute numbers of CD14+ monocytes were not different in both groups. In patients on HD the expression of the TNF-R2 (CD120b) was significantly enhanced on α/β TCR positive lymphocytes: mean MESF 5466 ± 892 versus 3151 ± 507 as well as on monocytes: mean MESF 10233 ± 3531 versus 2563 ± 808, p=0.01 and p=0.02, respectively. In contrast, the expression of the activation markers on the T-cell surface, CD25 (IL-2Rα), CD122 (IL-2Rβ) and HLA-DR, were comparable between HD patients and healthy controls (Figure 3).

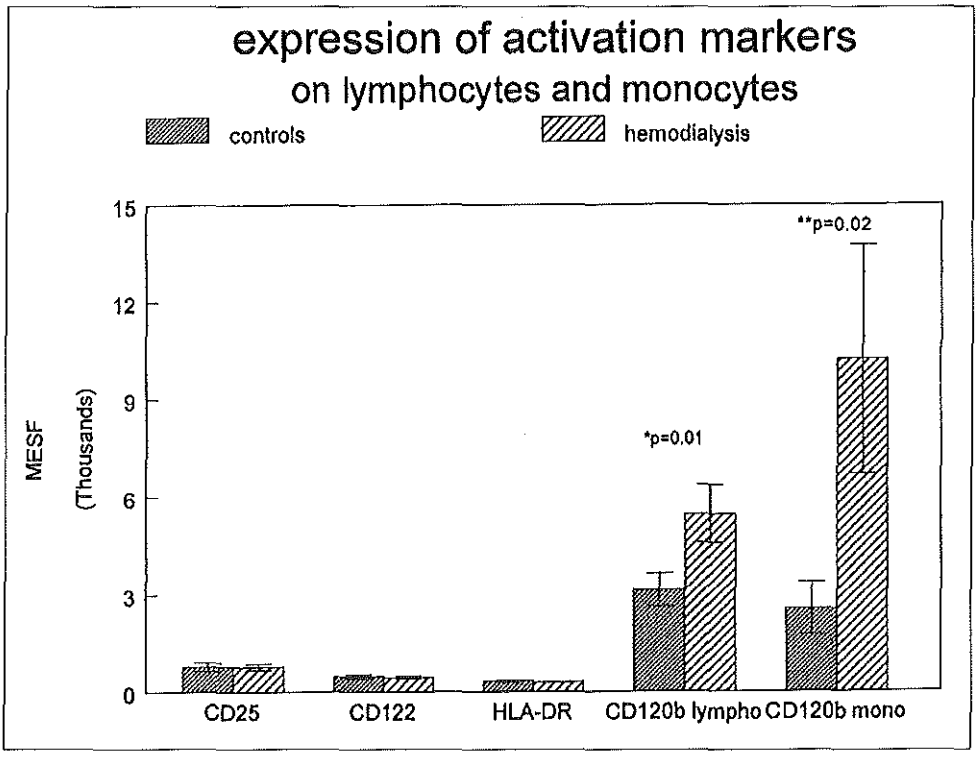

Figure 3:
Expression of activation markers.
IL-2Rα chain (CD25), IL-2Rβ chain (CD122) and HLA-DR on lymphocytes and TNF-R2 (CD120b) on lymphocytes and monocytes, measured by quantitative flow cytometry, denoted as MESF (Mean ± SEM).

Soluble TNF and IL-2 receptor levels by ELISA

TNF-α system

Plasma TNF-α protein levels were significantly higher HD patients compared to controls: 5.6 ± 0.9 pg/ml versus 0.9 ± 0.1 pg/ml, $p<0.001$. To test the TNF-α production capacity by T-cells we stimulated PBMC with the mitogen PHA. This resulted in significantly lower TNF-α concentrations in supernatants of PBMC from HD patients than in supernatants of PBMC from healthy controls: mean TNF-α: 220.6 ± 92.9 pg/ml versus 1012.2 ± 341 pg/ml, respectively, $p<0.001$. Correction for the lymphopenia in HD patients results in a mean TNF-α production of 331 pg/ml, which

still is significantly lower compared to the production of the T-cells obtained from healthy controls, p=0.04. The levels of sTNF-R1 were significantly higher in culture supernatants of HD PBMC compared to control PBMC: mean sTNF-R1 (after PHA-stimulation):115.0 ± 7.1 ng/ml (corrected for lymphopenia: 172 ng/ml) (HD) versus 78.3 ± 9.0 ng/ml (controls), p=0.008 (p<0.0001, corrected). After IL-2 stimulation the sTNF-R1 levels were: 107.4 ± 11.0 ng/ml (corrected: 161 ng/ml) (HD) versus 74.8 ± 6.7 ng/ml (controls), p=0.027 (corrected: p<0.0001). The sTNF-R2 production after PHA stimulation was not significantly different between patients and controls. IL-2 stimulation resulted in a higher production of sTNF-R2 in supernatants of HD patients: 461.3 ± 51.4 ng/ml (corrected: 692 ng/ml) versus 386.6 ±54.2 ng/ml (controls), p=0.0005 (after correction for lymphopenia). Table 1.

Table 1:
PHA and rhIL-2 (10 ng/ml) induced TNF-α, sTNF-R1 and sTNF-R2 production by PBMC obtained from hemodialysis patients and healthy controls, cultured in pooled serum, measured in supernatant after 3 days stimulation, by ELISA

		controls	patients	patients	p-value*
		n=11	n=15	corrected data[b]	
TNF-α	day 0	4.8 ± 1.2	4.8 ± 1.2		n.s.
[pg/ml]	PHA	1012.2 ± 341[a]	220.6 ± 92.9[a]	330.9 ± 92.9[a]	0.04
	IL-2	31.2 ± 8.7	18.2 ± 3.4	27.3 ± 3.4	n.s.
sTNF-R1	day 0	79.0 ± 1.4	79.0 ± 1.4		n.s.
[ng/ml]	PHA	78.3 ± 9.0	115.0 ± 7.1	172.5 ± 1.4	<0.0001
	IL-2	74.8 ± 6.7	107.4 ± 11.0	161.1 ± 11.0	<0.0001
sTNF-R2	day 0	88.3 ± 1.0	88.3 ± 1.0		n.s.
[ng/ml]	PHA	540.1 ± 94.9[a]	449.4 ± 94.7[a]	674.1 ± 94.7[a]	0.34
	IL-2	386.6 ± 54.2[a]	461.3 ± 51.4[a]	692.0 ± 51.4[a]	0.0005

Mean ± SEM
* Student's t-test
[a] p<0.001 versus day 0
[b] corrected for hemodialysis related lymphopenia
n.s. = not significant

IL-2 system

In HD patients and controls baseline plasma IL-2 levels were within the detection limit of the used ELISA test. In contrast, IL-2R (CD25) levels were significantly higher in plasma of HD patients: mean 1467 ± 230 U/ml versus 409 ± 13 U/ml, for healthy controls, $p<0.001$, table 2. After 3 days of PHA stimulation the IL-2 and sIL-2R (sCD25) production capacity of PBMC, when measured in own plasma was comparable between PBMC from HD patients and controls. However, when the assay was performed in pooled serum, the PHA-induced IL-2 production by PBMC was significantly higher in HD patients, $p=0.03$. The PHA-induced sIL-2R production was comparable in both groups, table 2.

To obtain an impression of the biological activity of the sIL-2R (sCD25), we performed an IL-2 recovery study. In plasma, the IL-2 recovery after addition of 125 pg/ml rhIL-2 was 55 ± 9.4% (HD) versus 43% (controls), $p=0.02$, and in the presence of surplus of rhIL-2 (500pg/ml) the recovery remained increased in 59.6 ± 9.4% versus 49.9 ± 8.2%, respectively, $p=0.05$, Mann-Whitney. Table 2.

Table 2:
Plasma levels at t=0 (baseline) and the PHA-induced production of IL-2 and sIL-2R by PBMC obtained from healthy controls and HD patients, measured in supernatant after 3 days of stimulation using ELISA. Tests are performed in own plasma and pooled serum.

	controls	patients	patients	p-value[b]
	n=11	n=15	corrected data[a]	
Baseline				
IL-2 [pg/ml]	9.3 ± 3.1	5.0 ± 0.0		n.s.
sIL-2R [U/ml]	409 ± 13	1467 ± 230		< 0.001
PHA-induced				
IL-2 [pg/ml] own	1440 ± 217	1956 ± 944	2934 ± 944	0.19
pooled	2823 ± 558	2996 ± 482	4494 ± 482	0.03
sIL-2R [U/ml] own	124 ± 33	93 ± 15	140 ± 15	n.s.
pooled	99 ± 23	86 ± 31	129 ± 31	n.s.
IL-2 recovery				
+ 125 pg rhIL-2	43.1 ± 8.4%	55 ± 9.4%		0.02
+ 500 pg rhIL-2	49.9 ±8.2%	59 ± 9.4%		0.05

Mean ± SEM
[a] corrected for dialysis related lymphopenia
[b]Student's t-test
n.s. = not significant

Proliferation tests

PHA and rhIL-2 stimulation resulted in a significantly increased proliferation of PBMC obtained from HD patients as well as from healthy controls. Proliferation assays performed in own plasma showed comparable proliferation capacity of PBMC from healthy controls and HD patients, when corrected for the HD-related lymphopenia, p=0.07. When performed in pooled serum the PBMC from healthy controls, as well as from HD patients, had a higher proliferation capacity, without differences between the two groups. Figure 4A. Also IL-2 induced proliferation capacity was, when results were corrected for the HD-related lymphopenia, comparable by PBMC obtained from healthy controls and HD patients, irrespective whether the test was performed in own or pooled serum. Figure 4B.

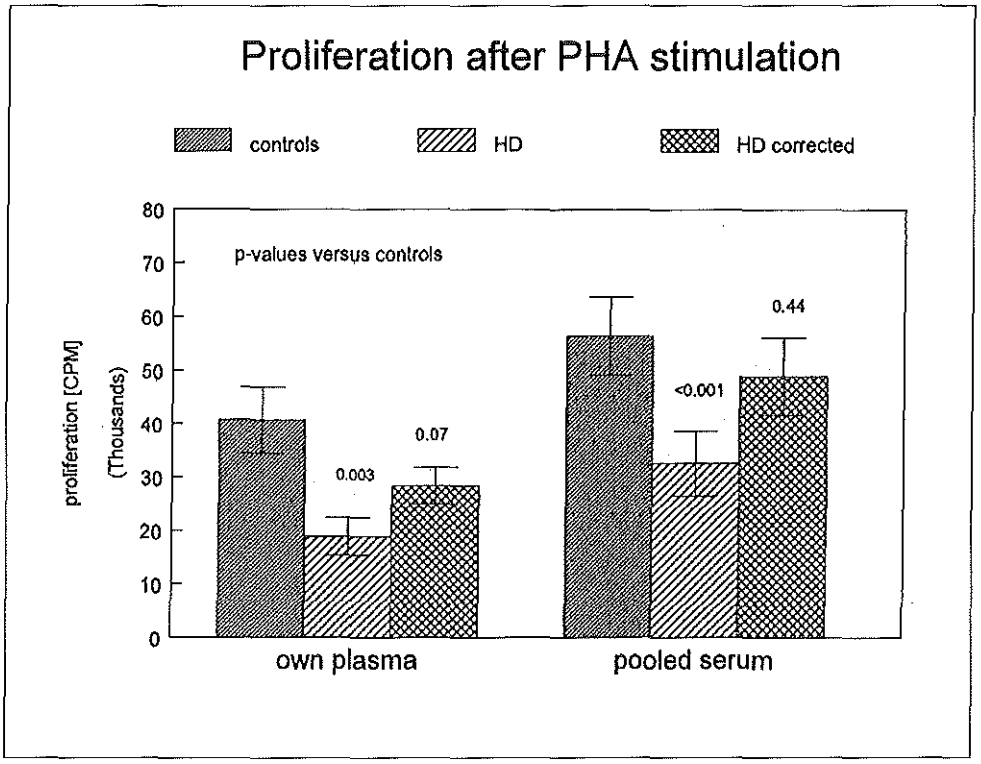

Figure 4A:
The PHA-stimulated proliferation capacity of PBMC obtained from patients on hemodialysis and controls in own plasma and pooled (healthy) serum. The right bar represents results after correction for HD related lymphopenia.

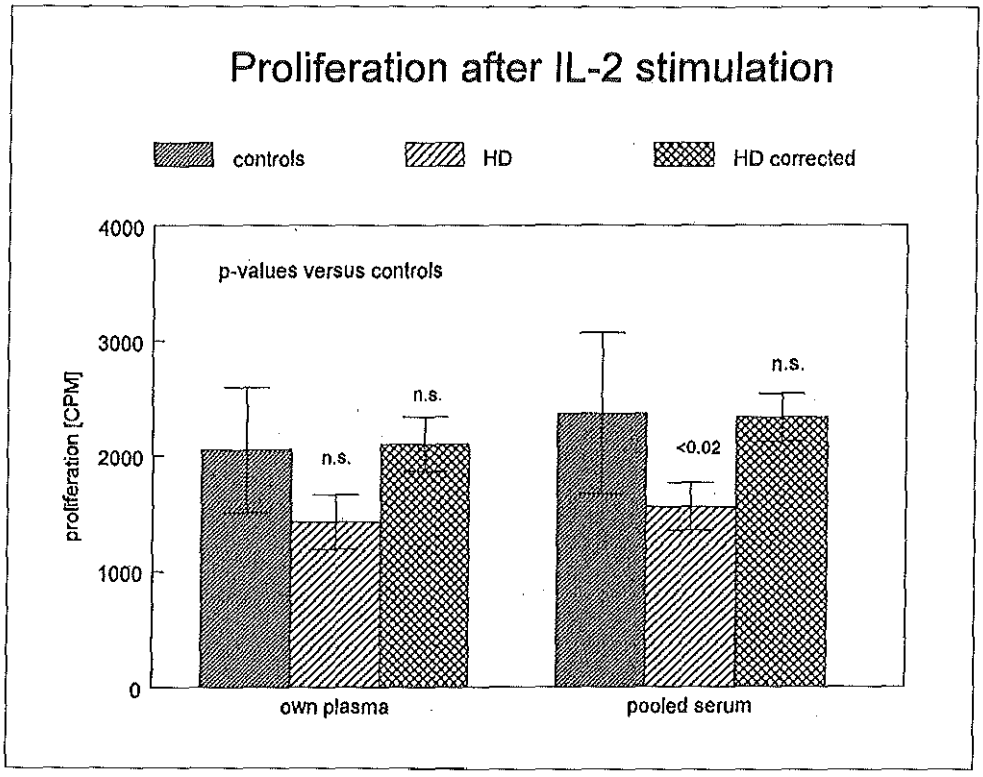

Figure 4B:
The IL-2 stimulated proliferation capacity of PBMC obtained from patients on hemodialysis and controls in own plasma and pooled (healthy) serum. The right bar represents results after correction for HD related lymphopenia.

Both the PHA- and IL-2 stimulated proliferation of PBMC from healthy controls was inhibited in uremic plasma. The mean relative stimulation index of the PHA-induced proliferation of healthy PBMC in normal plasma was 76 ± 9.0 versus 59.0 ± 8.0 in uremic plasma (p=0.16). The IL-2 induced proliferation resulted in a mean relative stimulation index of 93.5 ± 13.3 (normal plasma) and 68.0 ± 13.4 (uremic plasma), p=0.08.

Discussion

Immunologic function in hemodialysis patients is related to the overall survival in these patients (18). Cytokines, like TNF-α and IL-2, are important in the regulation of the immune responses. Imbalanced cytokine systems may result in ineffective immunological responses and consequently lead to high susceptibility for infections, malignancies and decreased responses on T-cell dependent antigens (1;2). In the present study, we measured the activity of both the TNF-α and the IL-2 system in patients on chronic hemodialysis, on mRNA and protein level as well as on membrane bound and soluble receptor level. Both systems are of paramount importance in the APC/T cell interaction initiating specific immune responses. Moreover, we performed functional assays to find out whether, *in vitro*, specific APC/T-cell defects were detectable in peripheral cells of HD patients.

The TNF-α system

In previous studies we detected significantly increased TNF-α mRNA expression by PBMC in patients on HD. Furhermore, we found high levels of active, soluble TNF-R, sTNF-R1 and sTNF-R2, which buffered recombinant TNF-α to a large extent (11). In the present study, we confirmed the activated state of PBMC by finding significantly increased receptor expression of TNF-α, TNF-R2 (CD120b) on both lymphocytes and monocytes of patients on chronic HD. Moreover, we were able to detect significantly higher levels of free plasma TNF-α, by using the sensitive ELISA kit. To evaluate the TNF-α production capacity by T-cells we measured PHA- and IL-2 induced TNF-α production, *in vitro*. In culture supernatants free TNF-α concentrations were significantly lower after PHA stimulation of PBMC from HD patients, in contrast to the IL-2 induced TNF-α. At the same time significantly higher concentrations sTNF-R1 and sTNF-R2 were found in the supernatants after stimulation, reflecting shedding of their increased receptors. From these data, we conclude that, although PBMC from patients on HD are constantly activated, T-cells are not able to produce adequate amounts of TNF-α upon stimulation.

Zamauskaite et al also described an impaired TNF-α production upon stimulation by T-cells, using intracellular cytokine staining, but only in patients on peritoneal dialysis therapy (19). The overproduction of TNF-α by PBMC of chronic HD patients reported by Girndt et al, is not contradictive to our results as they induced mononuclear cells with LPS, which stimulates monocytes rather than T-cells (12).

The IL-2 system

Chatenoud et al (20) suggested that the immunosuppressive state of HD patients was due to a deficient IL-2 system. However, conflicting data concerning parameters of the IL-2 system in patients with ESRF, on CAPD or HD, are present. Weinstein et al and Caruana et al reported increased levels of IL-2 in patients with ESRF and HD, while in patients on CAPD increased, as well as normal levels of IL-2 are found, all compared to healthy controls (21;22). Ha et al and Krisnamurthy et al demonstrated lower levels of IL-2 and a decreased IL-2 production in ESRF and HD patients (23;24). Rostaing et al describes the intracytoplasmatic expression of various cytokines, i.e. IL-2, IL-6, IFN-γ and TNF-α, in relation to different dialyzer membranes and compared the results to patients with pre-dialysis ESRF and healthy controls. By measuring the cytokines intracytoplasmatic he prevents difficulties in assessment of plasma cytokine levels and provides more insight in the influence of the various dialyzer membranes. It appeared from this study that patients dialyzed with a polysulphone membrane (PS) have cytokine profiles highly comparable to healthy controls (25). Our, *in vitro* data are in line with these findings. At both mRNA and protein level we measured comparable production of IL-2 between HD patients (dialyzed with PS and Hemophane membranes) and healthy controls. Also expression of the α-chain (CD25) and β-chain (CD122) of the IL-2R complex was not increased on T-cells in these HD patients. In previous work we have demonstrated no differences in T-cell activation markers at the single cell level in patients with ESRF, on CAPD and HD, using the quantitative flow cytometric analysis (15). So, no overt indication for an intrinsic deficient IL-2 system was established. However, significantly higher levels of sIL-2R (sCD25) were present in plasma from HD patients. These high levels of sIL-2R did bind up to 55% of rhIL-2, suggestive for an impaired biological availability of the produced IL-2.

Proliferation tests

In peripheral blood of patients on HD we found a significantly lower number of α/β positive T-cells. To evaluate the functional capacity of these T-cells we measured the PHA-stimulated proliferation capacity. This proliferation capacity of T-cells from patients on HD appeared to be significantly decreased, compared to control T-cells. These results are in line with Krisnamurthy, Raskova and Severini et al (24;26;27). However, when we corrected the data for the overt lymphopenia, found in HD patients, no statistically differences were present in the proliferation capacity of T-cells. The

influence of uremic environment was determined by comparing the proliferation capacity of PBMC obtained from healthy controls in uremic and pooled plasma. This proliferation capacity significantly decreased in uremic plasma, thereby confirming the data of Donati and Severini et al who studied the effect of uremic serum on PHA stimulated proliferative responses of healthy PBMC and purified T-cell sub-populations (27;28). The presence of a continiously activated TNF-α system and an inadequate TNF-α production upon stimulation suggest tachyphylaxis of T-cells. The IL-2 system seemed not intrinsically affected as T-cells were able to proliferate normally upon IL-2 stimulation. As in this present study, others have also attempted to link the TNF-α and IL-2 system (29). Owen-Schaub et al correlated the expression of TNF-R on lymphocytes to IL-2 production (30). According to these authors increased TNF-R expression indicated T-cell activation, whereas we assume that elevated TNF-R expression is independent of T-cell activation. Some publications described intrinsic T-cell dysfunction (31), while others reported normal T-cell function (14;32). T-cell dysfunction may be induced by the hemodialysis procedure (33-36), or result from monocyte dysfunction (9). Our data are in line with Modai, Degiannis and Kelly (8;14;37): an intact T-cell function, but a disturbed APC function, e.g. TNF-α production. Additional dose-response studies on cytokine-induced proliferation of normal T-cells in uremic plasma in the presence of antibodies against various immunoreactants could provide further evidence for our hypothesis. The results of this study can be influenced by the significantly older age of the HD patients compared to the age of the healthy controls, $p<0.001$. Many authors have described the influence of aging on cytokine levels and production. However, data on cytokine levels and production are not consistent. Concerning the pro-inflammatory cytokines, IL-1β, IL-6 and TNF-α, increased (38-40), as well as decreased (41;42) levels in the elderly are found. Bruunsgaard et al found lower levels of IL-1β and TNF-α in healthy old people (age: 81 years) when he compared the results to young men (age: 19-31 years). However, these differences disappeared when he compared the results of the elderly to that of young women (41)! The same conflicting data are described for IL-2, IL-4, IL-8 and IFN-γ (38;40;42-44). In our study the HD patients are significantly older compared to the healthy controls. However, in the the literature concerning about cytokine and aging, their mean age of 63 years is not regarded as old (in most studies old age is 80 years and older). We do not think that the differences in the studied parameters of the TNF-α and IL-2 system are influenced by the older age of the HD patients. In conclusion, the present study shows that the TNF-α system of PBMC in HD patients is

activated on all levels studied. We found increased expression of mRNA, high levels of free protein and soluble receptors and an overexpression of membrane bound receptors. Nevertheless, despite increased expression of TNF-α receptors, the TNF-α production capacity of T-cells is impaired, while other T-cell functions, e.g. their IL-2 production and proliferation capacity are intact. We hypothesize that this is due to tachyphylaxis resulting from continuous activation of the TNF-α system, rather than to an intrinsic T-cell defect.

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CHAPTER 5

Patients on chronic hemodialysis have no intrinsic T-cell defect upon stimulation with APC- and T-cell derived cytokines

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Summary

Patients on chronic hemodialysis suffer from general immune incompetence, resulting in high incidence of infectious complications, impaired response on vaccinations and a high incidence of malignancy. Multiple factors are involved in the defects of their immune system. Cytokines, which play a central role in the immune response are TNF-α and IL-2. Activated macrophages, shown by high expression of the membrane receptor, TNF-R2, and increased mRNA for TNF-α were found. A decreased PHA-stimulated TNF-α production by T-cells, together with significantly higher levels of biologial, active soluble receptors, resulted in a impaired bioavailability of TNF-α. Furthermore, no indication was found of T-cell activation, shown by no increased expression of the IL-2R or IL-2 protein. In this study, *in vitro,* cytokine-induced T-cell proliferation assays of T-cells obtained from patients on chronic hemodialysis were performed. Interleukine-2 (IL-2), interleukine-15 (IL-15) and TNF-α separately induced T-cell proliferation, however the combination TNF-α/IL-2 or TNF-α/IL-15 appeared to be synergistic in stimulation of the T-cell proliferation. The synergistic effects were more obvious in healthy controls compared to HD patients, but no differences were seen between both groups. Other macrophages-derived T-cell growth factors, like IL-15, play an additional role in T-cell proliferation and may compensate the impaired TNF-α production. We conclude that T-cells from HD patients show no intrinsic defects in proliferation capacity, *in vitro.*

Key words: interleukine-2, interleukine-15, TNF-α, T-cell proliferation, hemodialysis

Introduction

Patients on chronic hemodialysis develop an immunosuppressive state, resulting in a high incidence of infectious complications, a low response on T-cell dependent antigens, like hepatitis B and influenza vaccinations and a high incidence of malignant diseases [Kimmel et al, 1998; Maisonneuve et al, 1999]. The cause of this dialysis-related immune incompetence is not clarified. For initiating specific immune reponses three different signals are required [Male, Roitt, 1998]. The first signal is the antigen presentation by the antigen presenting cell (APC) in its MHC class II molecule to the T-cell receptor (TCR) on specific T-cells. Signal 2, the co-stimulation, is requisite for T-cells to become activated. Various co-stimulatory pathways have been identified: CD40/CD4L, CD28/$B7_{1-2}$. The third signal refers to the production of specific cytokines by the activated APC (i.e. IL-1, IL-6, TNF-α) [Feldmann, 1998]. Cytokines play a pivotal role in the APC-T cell interaction. The macrophage-derived IL-15 is a

potent T-cell growth factor [Weiler et al, 1998] and shares a number of biological functions with the T-cell derived IL-2 [Baan et al, 1999; Kennedy, Park, 1996]. TNF-α is mainly produced by monocytes, but T-cells can produce TNF-α as well, which can, in turn, be mediated by IL-15 [McInnes, Liew, 1998]. The HD-related immune incompetence may be related to imbalanced cytokine production by antigen presenting cells, T-cells, or by impaired co-stimulatory signaling pathways, or by intrinsic defects of APC and/or T-cells [Chatenoud et al, 1986; Girndt et al, 1995; Girndt et al, 1993; Donati et al, 1992]. Many studies have been performed to clarify these different aspects of the immune response in patients on chronic hemodialysis [Girndt et al, 1999]. Activated immunocompetent cells, unbalanced cytokine systems, i.e. TNF-α, IL-2, IL-6, have been described in hemodialysis, CAPD and in chronic renal failure [Lonnemann et al, 1990; Zaoui et al, 1991; Roccatello et al, 1993; Pereira et al, 1994; Engelberts et al, 1994; Descamps-Latscha et al, 1995; Pereira, 1995; Lin et al, 1996]. Both normal [Kelley, 1994] [Modai et al, 1990] and deficient T-cell functions are described [Kurz et al, 1986]. In previous studies we found a significantly lower PHA-stimulated TNF-α production by T-cells despite an overall activated TNF-α system. We concluded that the T-cells were tachyphylactic concerning the TNF-α production, but could be otherwise intrinsically normal [Van Riemsdijk-van Overbeeke et al, 2000a]. To test this hypothesis we analyzed proliferation of T-cells, derived from chronic HD patients upon stimulation with recombinant IL-2, IL-15 and TNF-α, and various combinations of these cytokines.

Patients and methods

Patients

From 9 patients (2 males, 7 females; mean age: 58 ± 4.1 years; mean creatinine: 923 ± 65 μmol/l) on chronic hemodialysis (mean time on dialysis: 45 ± 7 months) for 3 times/week, 4.5 hours/dialysis procedure, we collected peripheral blood at the start of the dialysis procedure. None of the patients suffered from infection or malignancy at time of blood sample collection or 3 months thereafter. They were all dialyzed using Hemophane dialyser membranes (MA-12H, Kawasumi Laboratory Inc., Minamiohi Shinagawa, Tokyo, Japan). All patients were on bicarbonate dialysate, no infectious moments of the dialysate were encountered (routine culures: $< 10^3$ micro organisms/ml). All patients were on subcutaneously administered recombinant erytropoietin and all received heparin during dialysis procedure. Three patients had

detectable antibody titers against hepatitis B, while 6 patients did not develop protective antibody titers in spite of multiple vaccinations. No patients were on immunosuppressive drugs. Reasons for renal insufficiency were hypertension (n=4), glomerulonephritis (n=3), reflux nephropathy (n=1) and IgA nephropathy (n=1). Ten healthy laboratory personel served as controls (5 males, 5 females, mean age:33.6 ± 3.5 years, creatinine < 100 μmol/l).

Methods:

Blood samples were collected in pyrogen-free tubes containing EDTA in a final concentration of 1 mg/ml at the start of the dialysis procedure. For the isolation of peripheral blood mononuclear cells (PBMC) the buffy coat was diluted in phosphate-buffered saline (PBS) solution and layered over a Ficoll-Isopaque gradient (δ = 1.077). PBMC for the *in vitro* assays were frozen and stored in liquid nitrogen.

After defrosting, the blood samples were monitored for the presence of lymphocytes with FACscan flow cytometry, using the monoclonal antibody, WT31 (Becton Dickinson, Mountain View, CA, USA), directed to the α/β chain of the T-cell receptor (TCR).

A dose-response curve of cytokine driven T-cell proliferation on rhTNF-α (5 to 100 ng/ml, Pepro Tech Inc, Rocky Hill, NJ, USA) was performed. Cytokine driven proliferation responses were tested by stimulating with rhIL-2 (10 ng/ml, Chiron, Amsterdam, The Netherlands), rhIL-15 (10 ng/ml, Serotec, Ltd, Oxford, UK) or a combination of 10 ng rhIL-2 plus 50 ng rhTNF-α or 10 ng rhIL-15 plus 50 ng rhTNF-α for 7 days at 37 °C in a humidified atmosphere containing 5% CO_2. Proliferation was measured after ^{3}H-thymidine (0.5μCi/well) incubation for 8 hours of culture before harvesting. Radioactivity was determined using a Betaplate counter (LKB, Bromma, Sweden) and data are expressed as counts per minute (CPM).

Statistics

All data are mentioned as mean ± SD. Comparison between proliferative responses of patients and controls were analyzed using the unpaired Student's t test, while proliferative responses on rhIL-2, rhIL-15, rhTNF-α and the combinations are analyzed in one group using the paired Student's t test. P-values ≤ 0.05 are considered significant.

Results

Control experiments:
Seventy percent of the PBMC were TCR α/β positive T-cells and 59% in samples from healthy controls, p>0.05. After stimulation with 10ng/ml rhIL-2 the percentage TCR α/β positive T-cells was 70% for HD patients and 67% for the controls, p>0.05. After incubation on 10 ng/ml rhIL-15: 75% (HD) and 69% (controls), p=0.34 and incubation on TNF-: 80% (HD) versus 77% (controls), p=0.55. After defrosting of PBMC the viability of the cells was tested by PHA stimulation. The mean CPM were 47,431 ± 24,599 (HD) versus 51,463 ± 25,080 (controls), p>0.05.

Cytokine proliferation assays:
The lymphocyte proliferation on rhTNF-α was not influenced by concentrations lower than 20 ng/ml rhTNF-α. TNF-α induced stimulation resulted in significantly increased proliferation at concentrations above 50 ng/ml. Figure 1. Therefore, the 50 ng/ml concentration of TNF-α was used in the simultaneously induction of T-cell proliferation with T-cell growth factors (i.e. IL-2, IL-15).

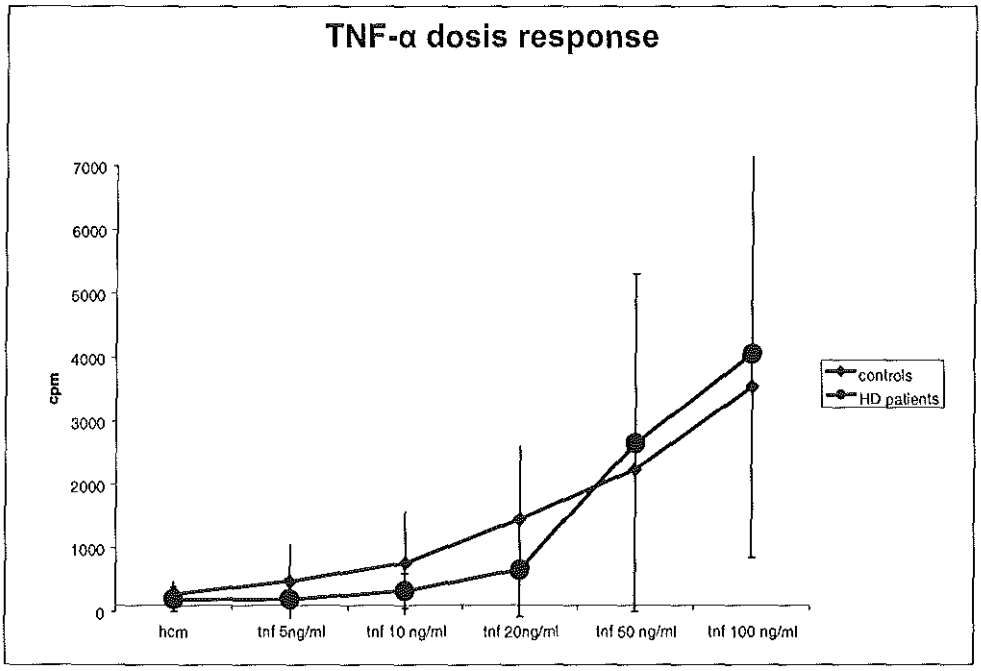

Figure 1:
T-cell proliferation on different doses of rhTNF-α (5 – 100 ng/ml) in hemodialysis patients (HD) and controls, depicted as counts per minute (CPM). Mean ± SD

The IL-2 induced proliferation capacity of T-cells, obtained from HD patients was comparable to the capacity of healthy T-cells: mean CPM: 23,781 ± 14,771 (HD) versus 22,578 ± 10,751 (controls). Also IL-15 induced proliferation was comparable in patients and controls: the mean proliferation capacity of T-cells was 3,334 ± 3,938 CPM (HD) versus 4,105 ± 3,256 CPM (controls). All data are reported as mean ± SD. Simultaneous stimulation with TNF-α and IL-2 or IL15 showed a higher increase in proliferation capacity than the increase of both cytokines alone. The combination rhIL-15 (10 ng/ml) and rhTNF-α (50 ng/ml) resulted in a mean ± SD CPM of 7140 ± 7058 (HD), increase of 19%, p=0.18. In controls the combination rhIL-15 (10ng/ml) and TNF-α (50 ng/ml) resulted in an increase of 57%, p=0.01. Also the combination rhIL-2 (10 ng/ml) and rhTNF-α (50 ng/ml) resulted in a proliferation capacity of 32,755 ± 17,955 CPM, an increase in HD patients of 24%, p=0.26. While in controls the

combination 10ng/ml rhIL-2 and 50 ng/ml rhTNF-α resulted in an increase of 55%, p=0.027. Figure 2.

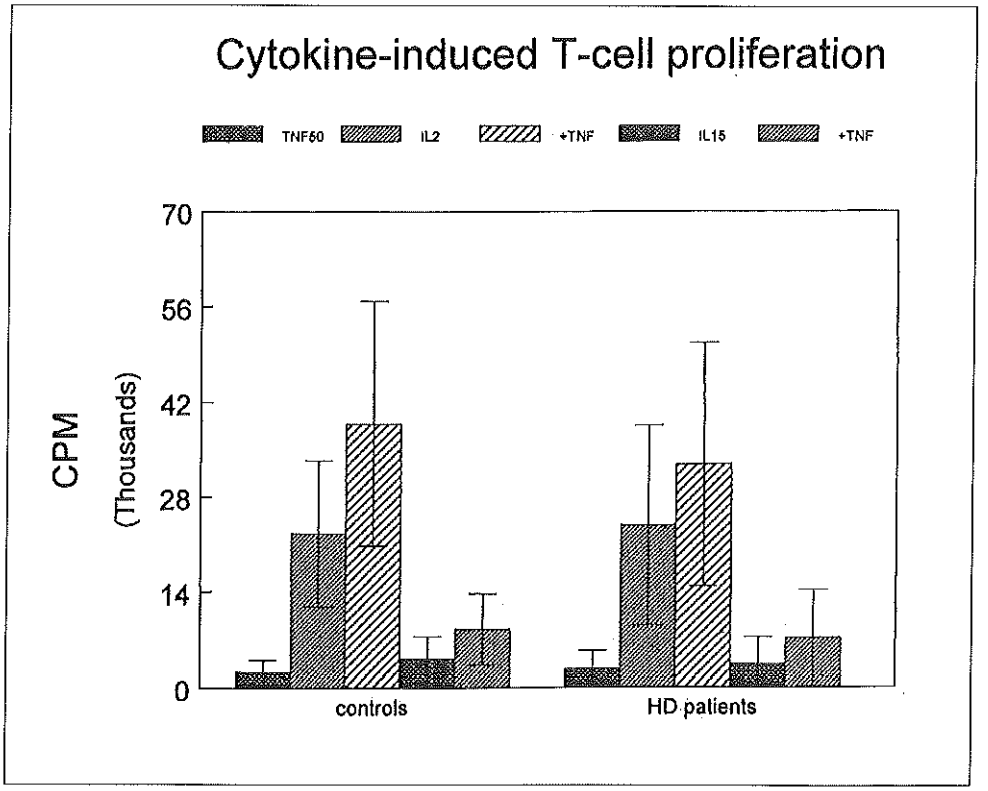

Figure 2:
T-cell proliferation after 7 days of stimulation with tumor necrosis factor-α (50 ng/ml=TNF50), interleukin-2 (10 ng/ml=IL2) or simultaneously IL-2+TNF-α (+TNF), interleukine-15 (10 ng/ml=IL15) or simultaneously IL-15+TNF-α (+TNF) in controls and in patients on hemodialysis (HD), depicted as counts per minute (CPM). Mean ± SD

In vitro, no significant differences were present in cytokine driven T-cell proliferation between HD patients and controls.

Discussion

Patients on chronic hemodialysis suffer from deficient immune competence resulting in multiple infectious complications, decreased response on T-cell dependent antigens, and even a pronounced increase in the incidence of malignant diseases [Kimmel et al, 1998; Maisonneuve et al, 1999]. This seems in contrast to the activated state of the monocytes and macrophages in peripheral blood [Van Riemsdijk-van Overbeeke et al, 2000b] [Le Meur et al, 1999]. T-cell functions, like mitogen induced IL-2 production [Chatenoud et al, 1986], proliferation capacity [Donati et al, 1992] were investigated in a number of studies. Some authors describe normal [Kelley, 1994; Modai et al, 1990], while others describe impaired T-cell functions [Descamps-Latscha et al, 1994; Deenitchina et al, 1995]. Cytokine production capacity for TNF-α and IL-2 were described, showing a lower production of specific "Th_1"cytokines (IL-2, TNF-α, IFNγ) in HD patients compared to patients on peritoneal dialysis [Zamauskaite et al, 1999] [Girndt et al, 1998]. In previous studies, we found a diminished TNF-α production by T-cells of patients on HD after mitogen (i.e. PHA) stimulation. In these patients the IL-2 mRNA and PHA-stimulated IL-2 production were comparable to that of healthy controls. We postulated a tachyphylactic state of T-cells concerning the TNF-α production, probably as consequence of continuous stimulation by blood-membrane contact or subclinical infections without an intrinsic T cell defect [Van Riemsdijk-van Overbeeke et al, 2000a]. Girndt et al [Girndt et al, 1995] described a possible defect in the co-stimulatory pathways, as he exposed the uremic T-cells to Raji cells (human Burkitt's lymphoma) and concluded that defects in T-cell proliferation of HD patients were located in the costimulatory pathway: B7/CD28. In the complex cytokine network interactions between cytokines are important.

In the present study we evaluated the proliferation capacity of T-cells, obtained from stable patients on long-term hemodialysis, after stimulation with rhIL-2, rhIL-15, and rhTNF-α in order to find out whether uremic T-cells are able to proliferate in the presence of adequate amounts of growth factors. We performed a TNF-α dose-response curve and found 50 ng/ml as optimal TNF-α concentration for our *in vitro* stimulation assays. There were no statistically differences in proliferation capacity of T-cells from HD patients or controls on TNF-α stimulation. Cytokine-induced T-cell proliferation on rhIL-2 (10ng/ml) and on rhIL-15 (10ng/ml) were comparable in HD and controls. IL-2 induction resulted in a stronger stimulation of the proliferation compared to induction with the macrophage-derived T-cell growth factor, IL-15. Simultaneous

stimulation of T-cells with the combination TNF-α/IL-2 or TNF-α/IL-15 appeared to be stronger than stimulation with the cytokines alone. Again, no differences were found in proliferation capacity on cytokines between lymphocytes from HD patients and controls. From these data we conclude that in patients on chronic hemodialysis no intrinsic T-cell defect, *in vitro,* is present. The tachyphylactic state for TNF-α production by uremic T-cells results in a lower cytokine driven proliferation capacity, *in vitro.* Another confounding factor is the uremic environment. This environment may strongly influence the function of T-cells. The results of our study confirm that lymphocytes from HD patients have no intrinsic defects and their proliferative capacity resembles that of "healthy" lymphocytes as long as adequate concentrations of growth factors are present.

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CHAPTER 6

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 hemodialysis patients. The imbalance between the high levels of soluble TNF-receptors RI 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 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 RI 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 their immunosuppressive therapy, recipients of a living related kidney do have a better balanced TNF-α system compared to hemodialysis patients.

Key words: TNFα, 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 in patients on haemodialysis, on 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 peripheral blood mononuclear cells (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/sTNF-receptor 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-R1 and sTNF-R2, and TNF-α mRNA before and after kidney transplantation.

Patients, material and methods

Patients

In 16 patients (9 male, 7 female; 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 hemodialysis, six patients on CAPD). Donations were from parent to child in eight cases and between siblings in eight cases (five identical, four haplo-identical 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 one 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 centrifugated, 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 centrifugated 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 (δ = 1.077). After centrifugation, the PBMC were removed from the interface, and washed twice with ice-cold PBS. Immediately following procurement, 2 x 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 limits. Especially at the lower levels the kits of 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). The sTNF-R1 and -R2 in plasma and urine were measured using double sandwich ELISA (R&D Systems Europe, Abingdon, UK). The detection limit of this commercial kit was 15 pg/ml for sTNF-RI and -R2. 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, WI) 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 house-keeping 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 mM Tris-HCl, pH 8.3, 50 mM KCl, 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 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, NJ) 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% phytohemagglutinin (PHA)-M (Difco, Detroit, MI) 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 analyzed 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, NY). 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 be equal to the known starting concentration of the competing internal control. The relative concentrations of TNF-α transcripts were divided by the relative concentration 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.

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 mean serum creatinine 171 µmol/l (range:76-265 µmol/l). The creatinine clearance was 59 ml/min. Patients using azathioprin 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 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.

Table 1:
sTNF-receptors (R1 and R2) and mRNA TNF-α before and after kidney transplantation

	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
pre-transpl	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					
cyclosporine	3.2 ± 1.3	1.8 ± 0.7	5.8 ± 1.8	5.8 ± 1.1	n.d.
azathioprine	2.5 ± 0.7	2.7 ± 1.1	4.0 ± 0.8	9.3 ± 1.8	n.d.

mean ± s.d.
n.d.: not done
* $p < 0.001$ versus controls and versus day 1; ANOVA
** $p < 0.001$ versus pre-transplantation

After transplantation sTNF-RI levels decreased to 3.8 ± 1.4 ng/ml ($p<0.001$) on day 1 and 3.0 ± 1.4 ng/ml ($p<0.001$) on day 6 (Table I). In spite of the restoration of renal function sTNF-RI levels remained significantly higher than those of healthy controls (0.7 ± 0.1 ng/ml; $p < 0.001$). sTNF-R2 levels showed also 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 I). 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-RI levels (3.2 ± 1.3 ng/ml) not significantly different from those in patients on azathioprine (2.5 ± 0.7 ng/ml; N.S.). sTNF-R2 levels were also not significantly different in cyclosporine treated patients than in patients on azathioprine (5.8 ± 1.8 ng/ml versus 4.0 ± 0.8 ng/ml). 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-RI levels were 10.9 ± 2.5 ng/ml on day 1 and 6.7 ± 2.9 ng/ml on day 6 (N.S.). 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 (N.S). After 1 year sTNF-RI levels in urine were 1.8 ± 0.7 ng/ml (cyclosporine group) and 2.7 ± 1.1 ng/ml (azathioprine group; N.S), while sTNF-R2 levels were 5.8 ± 1.1 ng/ml (cyclosporine) and 9.3 ± 1.8 ng/ml (azathioprine; N.S.).

We evaluated the urinary excretion of sTNF-R by calculating the fractional clearance of the receptors in relation to that of the creatinine. Six days after renal transplantation the fractional clearance of sTNF-RI was 9% and of sTNF-R2 5%. The fractional clearances of the sTNF-R decreased to 2% for both receptors at one 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 vs 2.3 ± 0.11; N.S.).

TNF-α mRNA

In the PBMC of the patients receiving a living-related kidney transplantation we measured TNF-α mRNA using the 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 levels of TNF-α mRNA in both patients and healthy controls. Levels of TNF-α mRNA as well as TNF-α/keratin ratio were not different in

patients compared to healthy controls (TNF-α mRNA: 11 fg vs. 18 fg.; TNF-α/keratin ratio: 284 vs. 528; N.S.). 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 cyclosporine, low grade 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 to 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 sTNF-R levels remained elevated in transplant recipients compared to 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 succesful 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 shedding of the 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 can not be explained by increased renal function.

Nevertheless, after succesful renal transplantation the imbalance in the TNF-α system in patients with renal failure is improved. However, compared to healthy controls the persisting imbalance suggests an ongoing immunocompromised state of these patients after transplantation.

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CHAPTER 7

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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Letter to the Editor: TNF-α in chronic heart failure
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Abstract

The two soluble tumor 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 the 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-receptors, using RT-PCR and ELISA and performed a TNF-α bindingcapacity analysis, quantitating the buffercapacity 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 bindingcapacity resulting in a decreased TNF-α bioavailability may contribute to an immunosuppressed state in these patients.

Key words: TNF-α, soluble TNF-receptors, heart failure, heart transplantation

Introduction

Tumor necrosis factor-α (TNF-α) is a pro-inflammatory cytokine with cardiodepressant properties [1]. In dilated cardiomyopathy, in end-stage ischemic 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 the TNF-α levels [8]. Excessive catecholamine production and generalized endothelial dysfunction in these patients may contribute to the activation of TNF-α, both at 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 erytrocytes and resting T-cells, have membrane-bound TNF-receptors R1 and R2, which regulate the biological activity of TNF-α. After binding with the receptor, proteolytic cleavage results in splitproducts, the soluble TNF-receptors R1 and R2. High levels of sTNF-receptors are found in patients with heart failure and after successful heart transplantation, indicating that produced TNF-α is bound to its receptors, resulting in the production of these splitproducts. The circulating sTNF-receptors can bind 1 to 3 free TNF-α molecules, thereby acting as a buffer for locally produced TNF-α [15]. Under physiological circumstances free TNF-α is in balance with its sTNF-receptors. However, under a variety of clinical conditions the TNF-α/sTNF-receptor ratio may be disturbed [16]. Apart from heart failure, conditions in which the TNF-α system is out of balance are sepsis [17] and compromised renal function (renal failure, haemodialysis) [18]. In sepsis the TNF-α production overcomes the buffercapacity of the sTNF-receptors and free TNF-α 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-α production by activated peripheral blood mononuclear cells, and impaired clearance of the receptors disturbs the balance between TNF-α and its receptors. Moreover, generalized arteriosclerosis and nephrotoxic immunosuppressive drugs cause impaired renal function and lead to a decreased clearance of the sTNF-receptors in both heart failure and cardiac allograft recipients. This results in a disturbed TNF-α/sTNF-receptor ratio.

In the present study, we evaluated the netto balance of the factors: production and clearance. Therefore, we analyzed the potential TNF-α 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 bindingcapacity for recombinant TNF-α in patients and control plasma, after incubation with free, active, recombinant human TNF-α.

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, range 53-390), we measured TNF-α mRNA expression of peripheral blood mononuclear cells, free plasma TNF-α and soluble TNF-receptors (sTNF-R1 and sTNF-R2). The patients suffered from heart failure, due to ischemic 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). These patients had been admitted to the 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 7 patients the left ventricular ejection fraction was determined by radionucleotide angiography. Mean left ventricular ejection fraction was 18% (range:15-20%) in 9 patients. The 2 patients with mitral valve stenosis, however had a left ventricular ejection fraction of 65-70%. Medication consisted of digoxin (n=5), nitrates (n=5), Ca-entry blockers (n=5), ß-blockers (n=2), diuretics (n=6) and ACE-inhibitors (n=9). Two patients used amiodarone.

TNF-α measurements were also performed in 15 cardiac allograft recipients (11 males, 4 females; mean age 56 years, range 21-67) with stable renal function (mean serum creatinine: 113 μmol/l; range 63-140 μmol/l, creatinine clearance: 90 ± 27 ml/min). 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 till 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 (range 85-320 ng/ml, median 145). Twelve healthy subjects served as controls (4 males, mean age 30 years, range 24-52, creatinine <100 μmol/l).

Sample preparation

Blood samples were collected in pyrogen-free tubes, containing EDTA in a final concentration of 1 mg/ml. The samples were immediately centrifugated, plasma and cell fraction were separated and plasma was stored at -80 °C until analysis. For the 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, 2x 10^6 cells were snap-frozen in liquid nitrogen and stored at -80 °C for Reverse Transcriptase Polymerase Chain Reaction (RT-PCR).

Isolation of mRNA and copy-DNA reaction

Total RNA was extracted from snap-frozen samples by a modification of the guanidinium method (Chomczynski [19]), described by Baan [20]. Cells were homogenized in 500 µl 4 mol/L 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 sodium acetate (pH 5.2) at – 20 °C for 18 hours. Precipitates were pelleted at 10.000xg 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 denaturated for 5 min at 80 °C and then chilled on ice. First strand cDNA synthesis was performed from the isolated RNA with 0.5 µg hexanucleotides (Promega Corporation, Madison, WI) and transcribed with 1000 U Moloney murine leukemia virus reverse transcriptase (Gibco-BRL, Gaithersburg, MD) at 42 °C for 90 minutes in a total volume of 100 µl. The reaction mixture consisted of 20 µl 5x MMLV-RT buffer (250 mmol/L Tris-HCl pH 8.3, 15 mM $MgCl_2$, 375 mM KCl), 5 µl (10 mM) dNTP, 400 U of RNAsin (Promega) and 10 µl 0.1M DTT.

Competitive template Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

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'). PCR 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 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 hematopoeitic cells [21]. Five µl cDNA sample and 5 µl of gene specific competitive templates were added to 90 µl PCR mixture containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each 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 in different dilutions to constant amounts of sample cDNA for competitive co-amplification with specific primers. (Figure 1A).

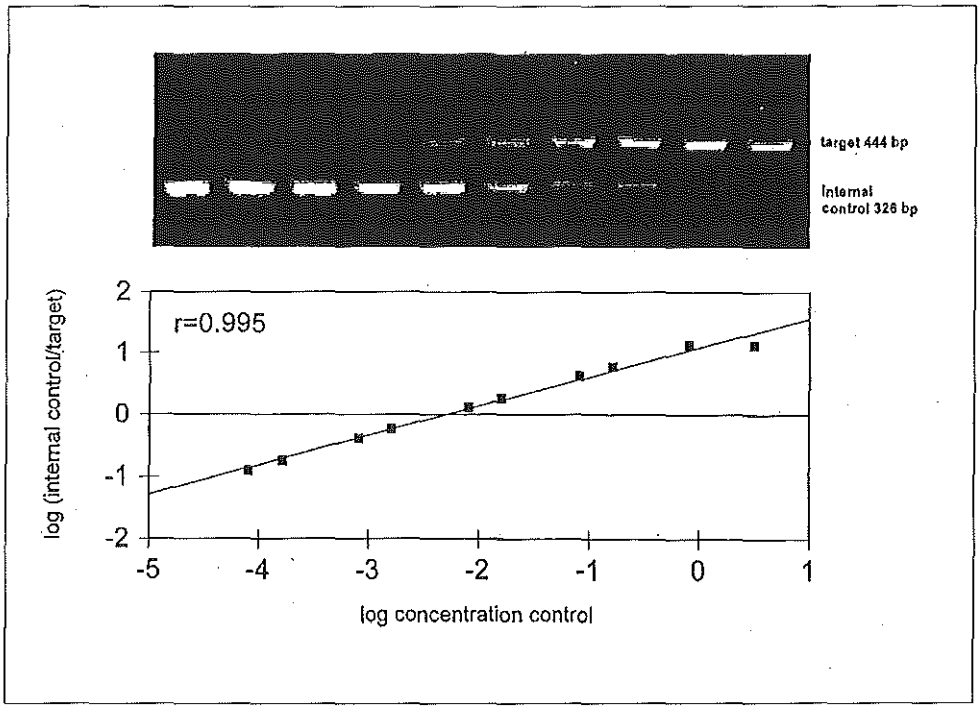

Figure 1A: (upper panel)
Typical example of quantitative RT-PCR analysis of TNF-α. After electrophoresis, the ethidium bromide stained agarose gel shows two PCR products of TNF-α per lane, representing the amplified target (444 bp) and internal control (326 bp)
Figure 1B: (lower panel)
The log of the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

The internal control was designed to generate a PCR product of a different size to allow differentiation between the amplified target and internal standard (TNF-α: 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) prior to PCR reaction in a DNA thermal cycler (Perkin Elmer- 480, Branchburg, NJ) 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% phytohemagglutinin (PHA)-M (Difco, Detroit, MI) 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 no-template reaction. Following PCR, 16 µl PCR product was analyzed by gel electrophoresis and the amount of products by the internal control and targets were determined for each individual 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). 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 TNF-α and keratin cDNA prior to PCR is assumed to be equal to the known starting concentration of the competing internal control (Fig. 1B). The relative concentration of TNF-α transcripts were divided by the relative concentration of keratin. This represents the amount of TNF-α 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-α in plasma was measured by a commercially available Enzyme Linked Immuno Sorbent Assay (Genzyme, Brussels, Belgium). The detection limit is 4 pg/ml, the coefficient of variation is 5-10%.

The plasma soluble TNF-receptors R1 and R2 are detected in the same blood sample, using double sandwich ELISA technique (R&D Systems Europe, Abingdon, United Kingdom). The detection limit for both receptors is 15 pg/ml, the coefficient of variation is less than 5% (sTNF-R1) and less than 10% (sTNF-R2).

Plasma TNF-α bindingcapacity 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). The plasma TNF-α bindingcapacity can be expressed as the TNF-α recovery. The 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 x 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 RT-PCR analysis to determine the level of TNF-α mRNA expression. Using the RT-PCR, 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 analyzed 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, Figure 2).

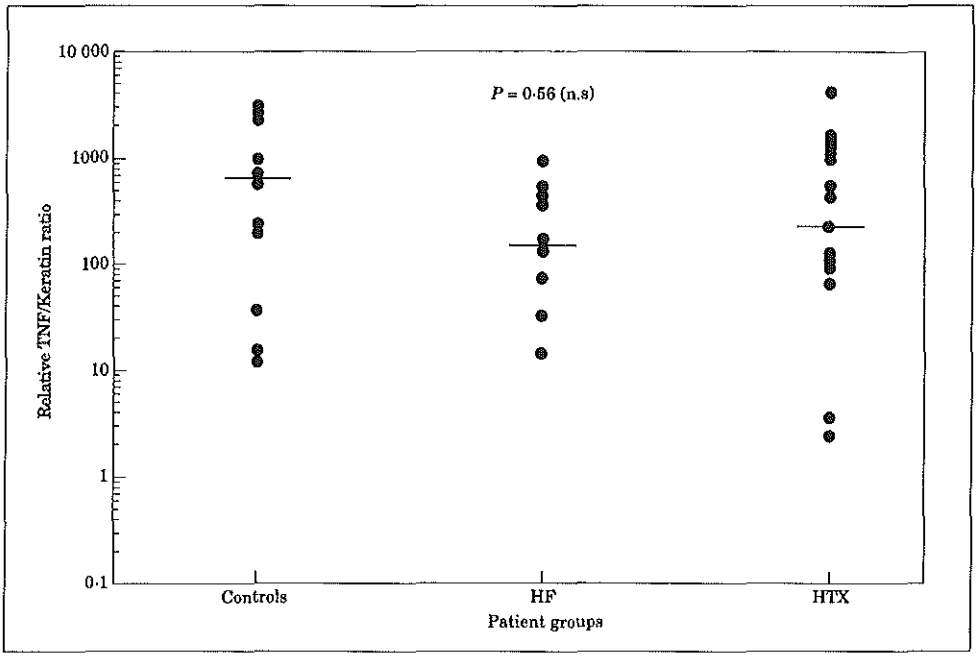

Figure 2:
TNF-α mRNA production by peripheral blood mononuclear cells obtained from patients with heart failure (HF), after heart transplantation (HTX), compared to healthy controls, denoted as the relative TNF-α/Keratin ratio, measured by competitive template RT-PCR.

This level of TNF-α mRNA-expression did not result in detectable TNF-α levels in plasma in either the patient groups or the healthy controls (all samples: TNF-α < 4.0 pg/ml, which is the lowest detection limit of the ELISA). However, significantly higher levels of sTNF-receptor R1 and R2 were found in plasma, obtained from patients with heart failure and 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. 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, and of sTNF-R2: 1.6 ± 0.5 ng/ml, $p<0.0001$; ANOVA. (Figure 3).

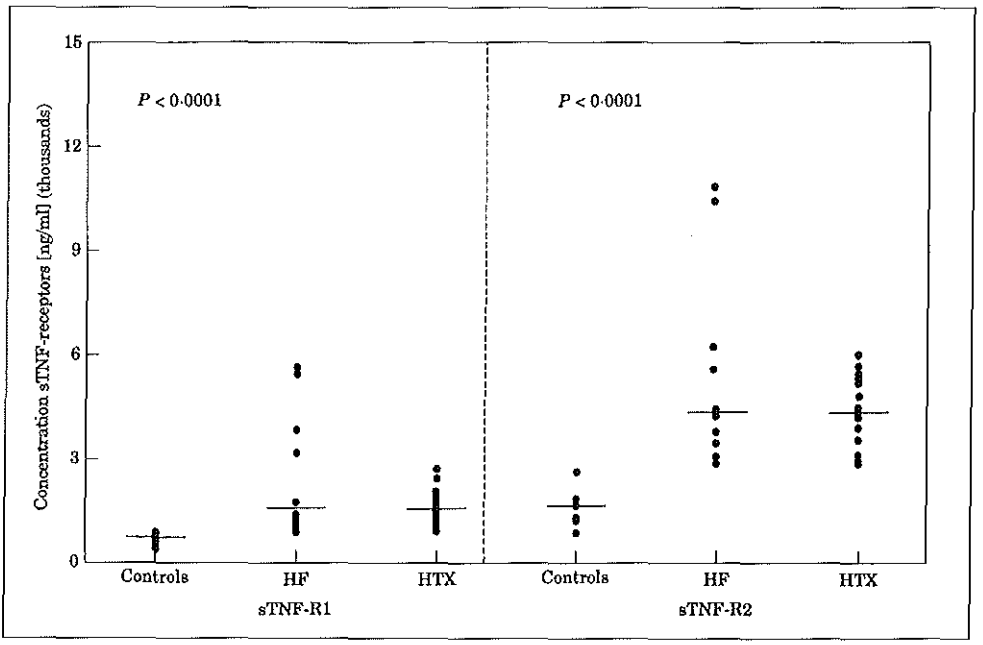

Figure 3:
Plasma levels of soluble TNF-receptors R1 and R2 [ng/ml] in patients with heart failure (HF) and after heart transplantation (HTX), compared to controls, measured by ELISA

Both sTNF-receptors showed a linear correlation with serum creatinine levels (sTNF-R1: r=0.92, sTNF-R2: r=0.82, p<0.001, Figure 4A + 4B).

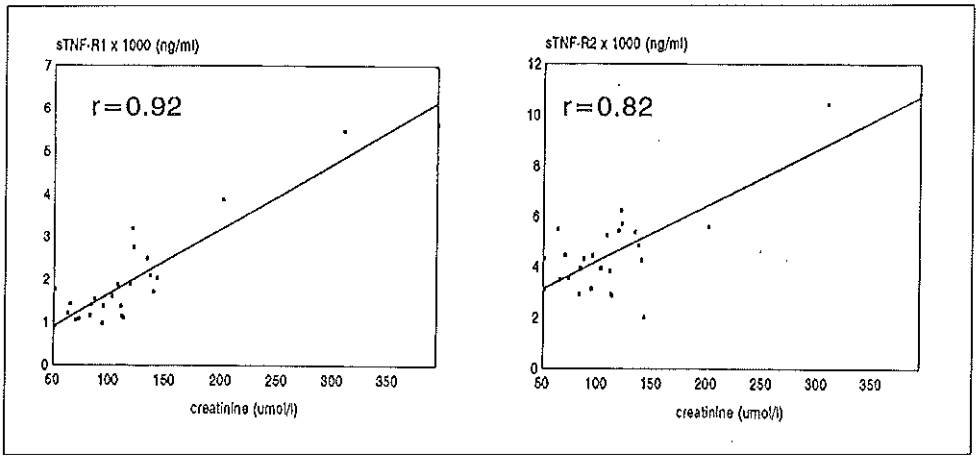

Figure 4A + 4B:
Correlation between serum creatinine (µmol/l) and soluble TNF-receptor R1 (ng/ml) [A] and R2 (ng/ml) [B] in patients with heart failure and after heart transplantation

Our results suggest that the absence of free, detectable plasma TNF-α is the consequence of the surplus of circulating sTNF-receptors. We, therefore, speculate that produced TNF-α is bound to the surplus of the circulating sTNF-receptors. To evaluate the biological activity of this surplus of sTNF-receptors we performed a TNF-α recovery test. A fixed amount of recombinant TNF-α was incubated with both patient plasma (heart failure and after heart transplantation) and control plasma. The TNF-α 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). Figure 5.

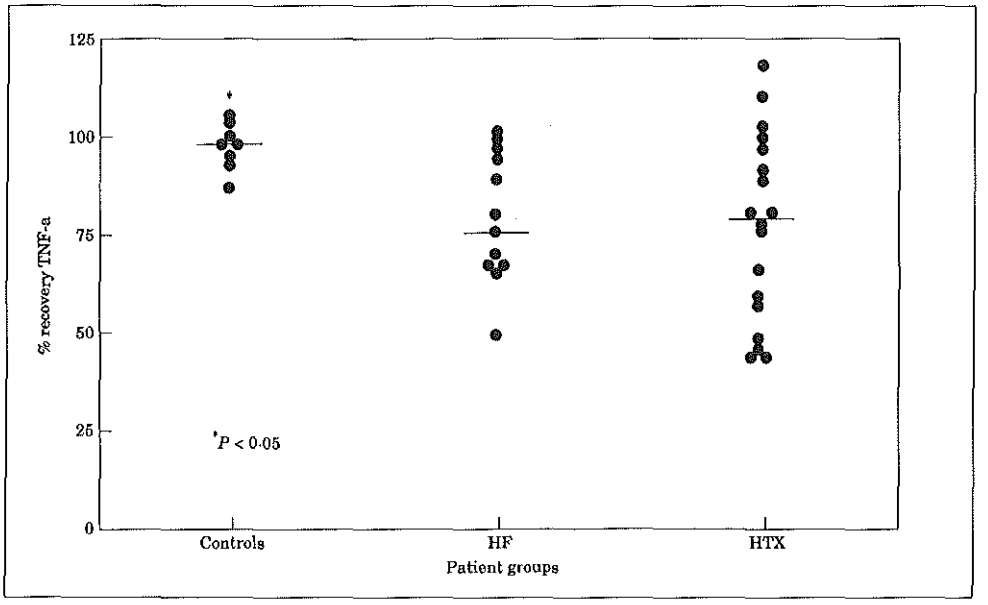

Figure 5:
TNF-α recovery, measured after incubation of 30 minutes of recombinant TNF-α in plasma of patients with heart failure (HF) and after heart transplantation (HTX) and control plasma, by ELISA, denoted as percentage of remaining TNF-α

Discussion

The TNF-α 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-α system may be the cause of higher susceptibility for infections and development of malignancies. In an attempt to asses the biological significance of the disturbed TNF-α system, we studied various components of the TNF-α 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 biological active soluble TNF-receptors. These sTNF-receptors are splitproducts of the TNF-membrane receptor, which are shed after TNF-α 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 result in a

cascade of events. Depending on interaction with R1 or R2, TNF-α binding leads to apoptosis, tumor cell lysis, hemorrhagic necrosis, T-cell proliferation or insulin resistence [24].

High amounts of TNF-α 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-α producers [25].

The lower detection limit of 4 pg/ml of our ELISA kit led us to anticipate finding TNF-α in the plasma from patients with heart failure. However, we detected no free plasma TNF-α. Commercially available ELISA kits show a wide range in measurement results, which make comparison between studies hardly possible [15]. Besides that, the high levels of sTNF-receptors were able to bind 25-30% of free, active TNF-α, *in vitro,* and thus could prevent measurement of free TNF-α. In previous studies we described the TNF-α 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-α expression (only in patients on haemodialysis) and high levels of sTNF-receptors. Uremia itself is known to be a potent initiator of TNF-α production, but in chronic haemodialysis, the blood-membrane interaction is seen as the main TNF-α production inductor. Impaired renal clearance has been found to give a positive correlation with the sTNF-receptor levels [23]. 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 in 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.4A+4B). Healthy controls reside below the regression line, suggesting that besides renal clearance, other factors, e.g. production, play a significant role. We found an unbalanced TNF-α system in patients with heart failure and after heart transplantation, caused by high levels of the 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 everpresent, smoldering TNF-α mRNA expression by graft infiltrating mononuclear cells, endothelium or cardiac myocytes 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 a high mortality from sepsis related side-effects, as described by Fischer et al [27]. In contrast, benificial 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-α 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-α system may play a central role. To overcome this decreased immunoreactivity, due to the imbalanced TNF-α system we have to increase clearance of the sTNF-receptors and thus decrease TNF-α buffercapacity of the plasma to a level, which is encountered in healthy controls.

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Letter to the editor:

TNF-α system in chronic heart failure

We appreciate the comments of Drs. Doehner and Anker regarding our paper about the TNF-α system in heart failure and after heart transplantation (Eur Heart J 1999; 20: 833-840). Their main concern is that we drew our conclusions from non-detectable TNF-α levels. In order to overcome this problem elegantly, we measured mRNA expression levels, which provide indirectly information about TNF-α production, and soluble TNF-receptors (sTNF-R), which are produced after TNF-α binding to their specific membrane receptor. Levels of sTNF-R can serve as reflection of this binding process and thus activity of the pro-inflammatory cytokine TNF-α. In the study we measured oedematous, as well as non-oedematous patients with chronic heart failure (NYHC III or IV, mean left ventricular ejection fraction 18%). Statistically there were no differences between patients with or without oedema in TNF-α mRNA expression levels or sTNF-R levels. In none of the patients (with heart failure or after heart transplantation) or controls free TNF-α was detectable. After reading the manuscript of Niebauer et al [1], we performed another TNF-α study in patients with severe heart failure and oedema. In this study we used the Quantikine high sensitivity ELISA kit for TNF-α (R&D Systems), with detection limit of 0.5 pg/ml. This ELISA kit was also used by Niebauer et al [1]. As positive controls we measured TNF-α plasma levels in renal transplant recipients, who received rabbit-Anti-Thymocyte-Globulin (r-ATG) as rejection treatment. Healthy volunteers served as controls. TNF-α levels were 349 pg/ml after r-ATG treatment, 1.2 pg/ml for healthy controls and 4.0 pg/ml in patients with heart failure, which did not significantly decrease after treatment. These data, especially the results of the patients suffering from heart failure, are in line with our previous study. We are not able to reproduce the data on TNF-α levels, found in healthy controls and in heart failure, as described by Niebauer [1]. Their levels of sTNF-R are comparable to our data. Moreover, it is known that due to various aspects, TNF-α measurements are hard to compare [2]. The sensitivity of the ELISA kit used in the previous study was low (detection limit 4 pg/ml). The TNF-α results, measured by the high sensitivity R&D ELISA kit, do not force us to change our conclusions. The

approximately 1000-fold excess of sTNF-R may interfere with the function of low free, bioactive TNF-α concentrations. Therefore, we think that measuring TNF-α mRNA by RT-PCR is a more reliable method of analyzing cytokine systems of chronic heart failure patients. Data of the TNF-α system in end-stage renal failure are reported in our article, which will be published soon [3].

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CHAPTER 8

Quantitative flow cytometry to measure the TNF-α and IL-2 system after heart transplantation

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Abstract

After heart transplantation a high incidence of infections and malignancies is found. Not only immunosuppression, but also intrinsic unbalanced cytokine systems, e.g. TNF-α and IL-2, can result in impaired immune competence and may have a role in these complications.

The aim of this study was to assess the activity of the TNF-α and IL-2 systems after heart transplantation. In peripheral blood we measured expression of activation markers of TNF-α (TNF-R2) and IL-2 (IL-2Rα, IL-2Rβ-chain) on monocytes and lymphocytes using quantitative flow cytometric analysis. TNF-R2 expression was significantly enhanced on monocytes and lymphocytes in patients after heart transplantation, while the expression of IL-2Rα and IL-2Rβ was not elevated. Increased TNF-R2 expression in peripheral blood after heart transplantation reflects an activated TNF-α system, leading to high levels of active sTNF-R, which impairs TNF-α bioavailability and consequently leads to immune incompetence.

Key words: TNF-α receptor, IL-2R, heart transplantation, immune incompetence

Introduction

After heart transplantation (HTx) infections and malignancies are frequently encountered. At the University Hospital Rotterdam 333 cardiac allografts were transplanted between 1984 and 1997. Approximately 10% (n=35) of these patients died within 12 months after transplantation. In 9 of the 35 the cause of death was infection (n=4) or lymphoproliferative disease (PTLD, n=5). At 5 years post transplant another 24 patients had died, fourteen of them of infection (n=3) or malignancy (n=11). Infection and cancer were the reason for mortality in the follow-up after 5 years post-transplant in 2 and 8 out of 52 deceased, respectively [2,4]. Our data are in line with the ISHLT registry data [9]. The cause of death was infection, including cytomegalovirus (CMV), 16.6% within 12 months and malignancy, including PTLD (24.4%) thereafter [8]. Whether the high incidence of infections and malignancies is the consequence of immunosuppressive agents or also of an intrinsically disturbed immune system has to be elucidated. Cytokines play an important part in the regulation of the host defense and immune responses. For example, TNF-α is a primary mediator of immune regulation, produced by immune competent cells, such as monocytes and lymphocytes. It is a central cytokine in the Antigen Presenting Cell (APC) system, which is required for T cell activation. IL-2 in turn, is an important cytokine for T-cell proliferation and differentiation. Disturbances in either cytokine system, TNF-α or IL-

2, result in ineffective defense mechanisms against infections or malignant diseases. In a previous study in peripheral blood of HTx patients we found high levels of biological active, soluble TNF receptors, sTNF-R1 and sTNF-R2 [15]. In the present study we have analyzed the expression of the TNF-α receptor2 (CD120b) on T-cells and monocytes and the IL-2 receptors (CD25 and CD122) on T-cells to evaluate the degree of activation of the TNF-α and IL-2 system. Moreover, we measured a general activation marker, HLA-DR on T-cells. Measurements were performed using quantitative flow cytometry.

Patients and methods

In peripheral blood of 11 cardiac allograft recipients (9 men, 2 women; median age: 55.1 years [range 43-67], median time after transplantation: 576 days [range 231-3975]) we measured receptor expression of the TNF-α and IL-2 systems. All patients were in good clinical condition, without overt signs of heart failure, infections or malignancy and received cyclosporine and prednisolon as maintenance immunosuppression. Blood samples were simultaneously taken at the time of endomyocardial biopsy. Histological analysis of these biopsies showed no rejection, according to ISHLT criteria [3]. Twelve healthy subjects served as controls (4 men, 8 women, mean age: 30 years [range 24-52]).

Flow cytometry analysis

Peripheral blood samples were collected in EDTA containing tubes and monitored for the presence of monocytes and lymphocytes. Surface markers were analyzed by two-colour flow cytometry after staining with monoclonal antibodies directed against CD14 (monocytes), WT31 as a marker for the α/β chain of the T-cell receptor (TCR), and CD25 (IL-2Rα), CD122 (IL-2Rβ), (Becton Dickinson, San Jose, CA, USA), HLA-DR (Immunotech, Marseille, France) and CD120b (TNF-R2, Serotec, Oxford, UK). Antibodies, except CD120b, were directly conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE). For CD120b we used a two-step staining. After the primary step with CD120b, cells were incubated with $F(ab)_2$ Goat-anti-Rat IgG PE. The staining procedure was performed by incubating 15 µl 1/100 diluted CD120b antibody with 100 µl blood (30 min, at 4 °C). After washing in Hanks' Balanced Salt Solution (HBSS, Gibco BRL, Paisly, UK) with 0.1% Bovine Serum Albumin (BSA, Sigma, St Louis, MO, USA) and 0.01% sodium azide (Merck, Darmstadt, Germany), the red blood cells were lysed by FACS Lysing Solution (Becton Dickinson). Samples

were centrifugated and washed in Cell Pack (TOA, Hamburg, Germany). Flow cytometric analysis was performed on FACscan flow cytometer using Cell Quest software (Becton Dickinson). From each tube 1000 events were measured. To compare various measurements in time the flow cytometer was calibrated using specific calibration beads (Calibration Beads Quantum 1000, Flowcytometry Standards Corp. San Jose, PR, USA). Each bead contains a known amount of fluorochrome. The intensity of the fluorescence is converted using Quick Cal program for quantum beads software to a standard curve. The intensity is denoted as molecular equivalents of fluorochrome, MESF. (Figure 1) .

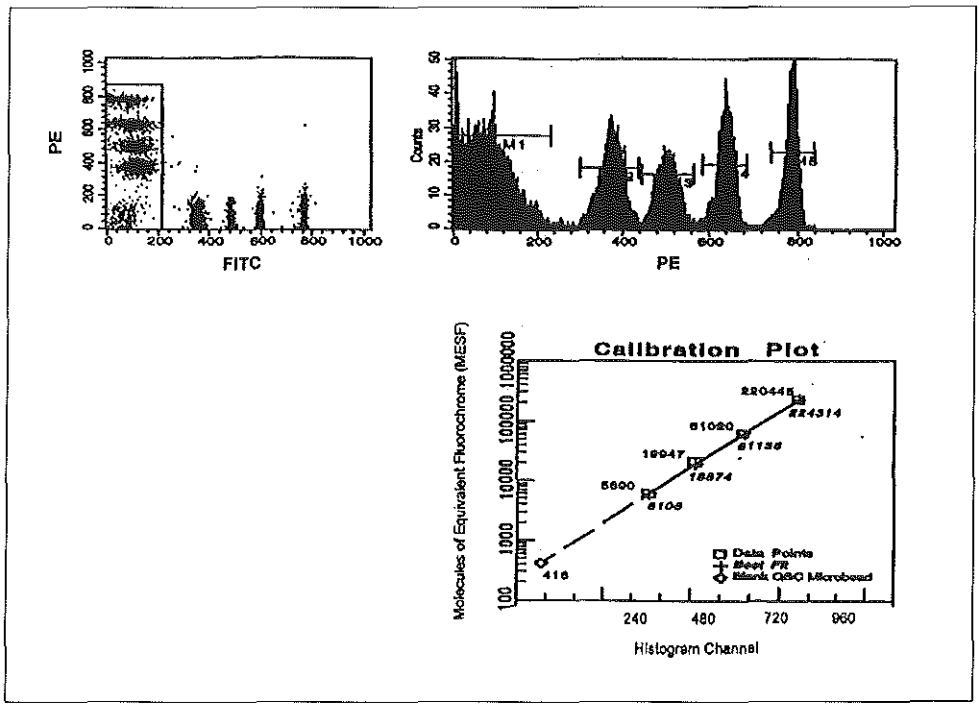

Figure 1:

Upper panel: Flow cytometric analysis of calibration beads, each of which contains a specific amount of fluorescine.

Lower panel: Standard curve of fluorescence intensity constructed after flowcytometric analysis of calibration beads, using Quick Cal program for quantum beads software. The intensity of the fluorescence is depicted as molecular equivalents of soluble fluorochrome (MESF)

Statiscial analysis

Data are presented as mean ± SD or median with range. The unpaired Student's t test was used to analyse the receptor expression data. P-values less than 0.05 were considered significant.

Results

Peripheral blood samples from patients and controls contained comparable absolute numbers of α/β TCR positive lymphocytes: patients: median 1094 cells/μl [range: 334-3104 cells/μl] versus controls: 979 cells/μl [range: 517-1773 cells/μl]. The absolute number of CD14 positive monocytes in peripheral blood was significantly higher in patients: median 468 cells/μl [234-735cells/μl] versus controls 314 cells/μl [range130-503 cells/μl], p=0.013. Quantitative flow cytometric analysis of the expression level of the T-cell activation markers, CD25 and CD122 showed no difference between patients and controls. CD25: MESF: 355 ± 4.4 versus 358 ± 4.2, p=0.59; CD122: MESF: 288 ± 4.9 versus 283 ± 3.6, p=0.43. In addition, the expression of the general activation marker HLA-DR on T-cells was comparable: MESF: 315 ± 6.5 versus 336 ± 7.5, respectively (p=0.13). In contrast, expression of the activation marker of the TNF-α system, TNF-R2 (CD120b) was significantly higher on both TCR α/β positive T-cells and monocytes from patients. On lymphocytes: mean MESF: 5733 ± 3409 versus 3078 ± 1935, p=0.032, and on monocytes: MESF: 6220 ± 2091 versus 2563 ± 808 (p=0.023, Figure 2).

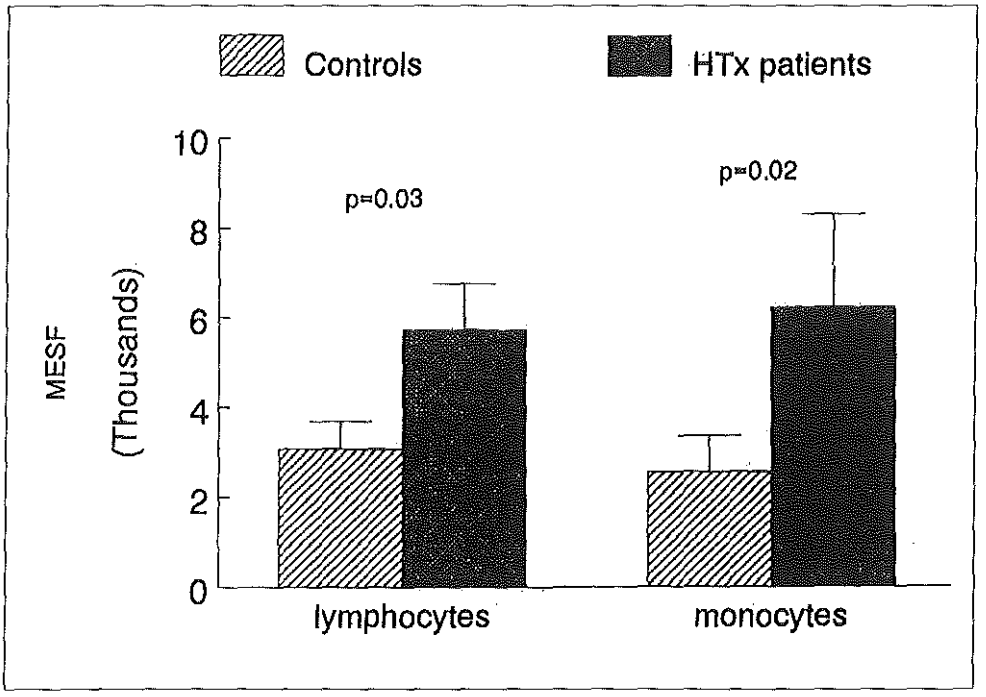

Figure 2:
Expression of the TNF receptor 2 (TNF-R2 = CD120b) on lymphocytes (left) and monocytes (right) in patients after heart transplantation (HTx) compared to controls, using qauntitative flow cytometry. The fluorescence is denoted as Molecular Equivalents of Soluble Fluorochrome (MESF)

Discussion

After heart transplantation high levels of cytokine activity can be detected in endomyocardial biopsies as well as in peripheral blood [5,6,10]. However, no clear relation between peripheral cytokine patterns and intragraft cytokine expression was found [7,10]. Within the transplanted heart the TNF-α system seemed to be continuously activated, while IL-2 mRNA expression was clearly related to rejection [1]. In peripheral blood of cardiac allograft recipients high levels of sTNF-receptors and sIL-2 receptor were reported, but these elevated levels were not consistently related to rejection [11,13]. In contrast, a correlation between cytokine activation and cardiac hemodynamic parameters is described [8,14], as well as between TNF-R and

clinical outcome after heart transplantation [5]. In the present study we measured the degree of activation of the TNF-α and IL-2 systems in peripheral blood by flow cytometric analysis. Increased expression of the TNF-R2, on both monocytes and lymphocytes, indicates an activated peripheral TNF-α system. In our previous study we found no upregulation of TNF-α mRNA in peripheral blood mononuclear cells, which is in contradiction with peripheral TNF-α activation. On the other hand, the high levels of sTNF-R, again, support the idea that the TNF-α system is activated after heart transplantation. Indeed, we described a constitutively TNF-α mRNA expression in endomyocardial biopsies after transplantation [1]. These data and our current results support our hypothesis that TNF-receptor expression on PBMC is induced by intragraft TNF-α production. In contrast to our TNF-R2 findings, we did not find an increased expression of the activation markers of the IL-2 system on peripheral blood cells suggesting that the IL-2 system is not activated. Free plasma TNF-α or IL-2 could not be detected, probably due to the high levels of soluble receptors of these cytokines. Previously, we showed that high sTNF-R levels impair the bioavailability of TNF-α [15]. This, consequently, may influence T-cell activation and result in an decreased defense mechanism against infections and malignancy. We postulate, that the transplanted heart, as source of continuous TNF-α production, might very well function as a central cause of the disturbed cytokine system and forms, together with the use of immunosuppressive agents, a continuous source of further immune suppresssion after heart transplantation.

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CHAPTER 9

Summary and Conclusions

Summary

In this thesis we have tried to clarify the role of the TNF-α system in the immune incompetence of patients with organ failure and after transplantation.

In **chapter 2** a unbalanced TNF-α system is found in patients with progressive renal failure. At first no free TNF-α was detected. When a more sensitive ELISA kit is used, significantly increased circulating TNF-α levels in HD patients are found compared to ESRF and CAPD. This indicates an activated TNF-α system, especially in HD patients. In contrast, no overt activation of TNF-α is found in patients on CAPD or with pre-dialysis ESRF. The dialysis procedure, itself, rather than the uremia, seems to activate the TNF-α in HD. Activation of TNF-α results in the production of sTNF-R1 and sTNF-R2. Due to decreased renal clearance high levels of these soluble receptors are found. Recovery studies reveal biological activity of these soluble receptors, which are capable of binding up to 75% of the added free TNF-α and thereby inhibiting the bioactivity and bioavailability of produced TNF-α. This unbalanced TNF-α system plays a role in the immune incompetence of patients on HD and their high susceptibility for infections and malignancy.

In **chapter 3** activation of the TNF-α system at the single cell level in HD patients is demonstrated. The reproducable quantitative flow cytometry technique was used to quantitate the amount of cell surface expression of the TNF-α receptor, TNF-R2 (=CD120b) on immune competent cells, monocytes and lymphocytes. The higher expression levels of the TNF-R2 reflect the activated state of the cells involved. Cytokines secreted by activated monocytes mediate the activation of T-cells. However, in spite of the activation of the TNF-α system the IL-2 system is not activated. This is shown by comparable expression of the IL-2R, IL-2Rα (CD25) and IL-2Rβ (CD122), on lymphocytes from HD patients and healthy controls. This strongly suggests that the T-cells are not activated and that the interaction between APC (TNF-α) and T-cells (IL-2) is disturbed. In contrast to the levels of soluble TNF-R, the expression of the

membrane-bound TNF-R2 did not correlate with renal function, supporting the hypothesis that renal failure itself does not induce cytokine membrane receptor expression.

In **chapter 4** *in vitro* stimulation of T-cells is described in order to find a possible intrinsic T-cell defect in HD patients. Again, an activation of the APC is found, without signs of activated T-cells. TNF-α is increased on the mRNA level as well as on the protein level, membrane-receptor expression and soluble receptor level. IL-2 is not increased on mRNA level or plasma level. The sIL-2R levels are also significantly increased in HD patients, due to decreased renal clearance. These sIL-2R are biological active, as are the sTNF-R. The proliferation capacity of T-cells from HD patients is lower compared to controls. However, when the data are corrected for the HD-induced lymphopenia no differences are present in proliferation capacity of T-cells. The influence of uremic plasma on proliferation capacity of PBMC obtained from healthy controls shows inhibition on the proliferation capacity, however not significant. Significantly less TNF-α production is found upon PHA stimulation in HD patients, in spite of high mRNA expression for TNF-α and high TNF-R expression on T-cells. We conclude that continuous stimulation of T-cells leads to tachyphylaxis for TNF-α production and that no intrinsic T-cell defect is present.

In **chapter 5** the proliferation capacity of T-cells obtained from HD patients on PHA, TNF-α, IL-2 and IL-15 stimulation is evaluated. It appeares that these cytokines are able to induce T-cell proliferation. When TNF-α concomitantly was given with IL-2 or the macrophage-derived T-cell growth factor IL-15, T-cell proliferation is augmented compared to stimulation of one cytokine alone. Proliferation capacity of T-cells obtained from HD patients is comparable to the proliferation capacity of T-cells from healthy controls. These data show that T-cells from HD patients have no intrinsic defect.

In **chapter 6** the course of TNF-α production and its soluble receptors, sTNF-R1 and sTNF-R2, is followed after successful renal transplantation. TNF-α is not detectable before and after transplantation. The levels of sTNF-R1 and sTNF-R2 are significantly increased before transplantation, and decrease dramatically after the transplantation, in concordance with increasing renal function. But despite recovery of the renal function the levels of the soluble receptors remained significantly increased compared to healthy

controls. After one year, the levels of soluble receptors, sTNF-R1 and sTNF-R2, are still significantly increased compared to controls, irrespective whether the patients are on cyclosporine or azathioprine as maintenance immunosuppressive therapy. After one year the creatinine clearance of patients on cyclosporine therapy is 59 ml/min, in contrast to patients on azathioprine: 84 ml/min. The fractional clearance of sTNF-R1 is 9% and of sTNF-R2 5%, in the first week after transplantation. The fractional clearance of both soluble receptors decreases to approximately 2% after one year. Messenger-RNA expression for TNF-α is comparable in patients before and during 6 days after transplantation. This shows an ongoing unbalanced TNF-α system, despite of recovery of renal function.

In **chapter 7** the actual activity of the TNF-α system in patients suffering from severe heart failure (HF) and in patients after heart transplantation (HTx) is studied. Messenger-RNA expression levels for TNF-α are comparable in PBMC derived from patients with HF, after HTx and from controls. The peripheral TNF-α system in HF and after HTx is not activated, shown by this normal mRNA expression. Detectable TNF-α protein levels are present in HF, when a sensitive ELISA kit is used, while after HTx and in controls no detectable TNF-α protein levels are present. In contrast, the soluble TNF-receptors, sTNF-R1 and sTNF-R2, are significantly increased in both HF and after HTx and thereby reflecting TNF-α activation. These high levels of sTNF-R are biological active and bind 21% (HF) and 25% (HTx) of free added TNF-α, resulting in a decreased bioavailability of produced TNF-α. In HF and HTx patients the TNF-α activation takes place in the failing or transplanted heart. Infiltrating immune competent cells i.e. lymphocytes, monocytes, but also activated myocytes or endothelial cells may produce and secrete TNF-α. The everpresent mRNA expression for TNF-α in endomyocardial biopsies supports these findings.

In **chapter 8** the activation of the TNF-α and IL-2 system, using the quantitative flow cytometry is measured. In peripheral blood of patients after HTx comparable numbers of T-cells are present, in contrast to absolute numbers of monocytes, which are significantly higher in these patients. Activation markers of the IL-2 system, the IL-2Rα (CD25) and IL-2Rβ (CD122) on T-cells are comparable between patients and controls, suggestive for non-activated T-cells. In contrast, the expression of the activation marker for TNF-α, TNF-R2 (CD120b), on monocytes as well as on lymphocytes is significantly increased in patients after HTx. In addition to chapter 7,

we conclude that the TNF-α system patients after heart transplantation is activated, and that most likely, this activation takes place in the graft. The transplanted heart functions as a central source of continuous TNF production, resulting in high levels of TNF-R, and in biological active soluble receptors, which prevent systemic TNF-α effects and thereby consequently contribute to further immune suppression in those patients.

Conclusions

From this study we conclude that the immune incompetence of patients on chronic hemodialysis is the result of impaired bioavailability of TNF-α in combination with a HD-related lymphopenia.

We found a chronic activated TNF-α system, shown by high expression of TNF-α mRNA, increased levels of TNF-α protein, high expression of TNF-R on the membranes of immune competent cells, i.e. lymphocytes and monocytes, and high levels of biological active sTNF-R. This in combination with a reduced TNF-α production capacity by T-cells leads to a decreased bioavailability of TNF-α.

We did not find an intrinsic T-cell defect, but there is a marked lymphopenia in patients on HD, which consequently leads to an impaired T-cell function.

So, the coincidence of low TNF-α bioavailability and a reduced number of T-cells result in an inadequate immune response in HD patients.

Patients with end-stage renal failure have no activation of the TNF-α system, suggesting that uremia alone is not responsible for the activation of the TNF-α system.

Patients on CAPD are comparable to patients on HD in respect to their renal function, but no increased mRNA expression for TNF-α is present in peripheral blood mononuclear cells. The high expression of the membrane-bound TNF-R on both lymphocytes and monocytes are nevertheless suggestive for an activated TNF-α system, and again due to impaired clearance of sTNF-R in CAPD patients the bio-availability of TNF-α is reduced.

The continuous activation of the TNF-α system is the result of the blood-membrane contact in HD patients, while in patients on CAPD it is caused by low-grade peritoneal infections of inflammation.

Nevertheless, the activated TNF-α does not result in activation of T-cells, in any of the patient groups. From these results we conclude that the interaction between APC (TNF-α system), and the T-cells (IL-2 system), is disturbed as result of the impaired TNF-α bioavailability.

In patients suffering from heart failure, the TNF-α system is also activated, but the ratio between TNF-α and the sTNF-R is less disturbed compared to the ratio TNF-α / sTNF-R in renal failure, because of normal renal clearance of the sTNF-R. The high levels of TNF-α, probably due to frequent bacterial infections from the gut, may lead to further deterioration of the heart function.
After organ transplantation, heart or kidney, the TNF-α system, appears to be constantly activated, reflected by increased expression of TNF-R on the membranes of immunecompetent cells. Due to immunosuppressive therapy the T-cell function is compromised. The combination of inhibition of T-cell function and a constantly activated TNF-α system leading to high levels of biological active sTNF-R results in a further immunosuppressive status of patients after organ transplantation.

We conclude that:

1. the TNF-α system in patients with renal and/or heart failure, and in patients after kidney or heart transplantation is activated
2. as the result of elevated levels of biological active sTNF-R the bioavailability of TNF-α is impaired, further contributing to the immunosuppressive status of these patients
3. HD patients have no intrinsic T-cell defect

CHAPTER 10

Samenvatting

Patiënten met een eindstadium orgaanfalen, zoals bijvoorbeeld nierfalen, hebben een verminderde afweer. Hierdoor zijn zij gevoeliger voor infecties, hebben een slechte response op vaccinaties, zoals tegen hepatitis B of influenza en hebben een hogere kans op het krijgen van kwaadaardige ziekten[1]. Dit lijkt in tegenspraak met de geactiveerde staat, waarin een gedeelte van hun afweercellen zich bevindt[2]. Ons immuunsysteem is ontwikkeld om ons te beschermen tegen ziekmakende factoren (antigenen). De cellen die de immuniteit onderhouden zijn de witte bloedcellen, zoals lymfocyten en monocyten, macrofagen en natural killer cells[3]. De immuunreactie bestaat uit drie verschillende fasen (signalen) met als doel de ziekmakende factor te elimineren. De eerste fase is de antigeen presentatie, hierin wordt het antigeen door zogenaamde antigeen presenterende cellen, APC, aangeboden aan lymfocyten. Het antigeen bevindt zich in een membraan gebonden molecuul, MHC klasse II op de APC. Op de T-lymfocyt zit de receptor, die het antigeen herkent. Het tweede signaal heet de co-stimulatie. Dit signaal is nodig om de T-cel te activeren. Na activatie zullen bepaalde stoffen, cytokinen, geproduceerd worden, die het verdere beloop van de immuunreactie verzorgen[4] (signaal 3). De belangrijkste cytokinen in deze reactie zijn IL-2 en TNF-α. Cytokinen werken via specifieke receptoren, die op de membraan van hun doelwitcellen zitten. Voor IL-2 is dat de IL-2R op lymfocyten en NK-cellen. Deze IL-2R bestaat uit drie ketens, de α-, β- en γ-keten. Deze ketens worden ook wel de CD25 (α-keten), CD122 (β-keten) en de CD132 (γ-keten) genoemd. Na binding van IL-2 op zijn receptor gaat de T-cel prolifereren en differentiëren. Daarnaast heeft IL-2 invloed op de cytolytische activiteit van NK-cellen en is IL-2 betrokken bij apoptose, geprogrammeerde celdood. Voor TNF-α zijn twee receptoren bekend, de R1 (ook wel p55 of CD120a) en de R2 (ook wel p75 of CD120b). Deze receptoren bevinden zich op alle lichaamscellen, behalve erytrocyten en rustende T-cellen. Na binding van TNF-α ontstaat productie van enzymen en adhesiemoleculen, terwijl binding via R2 (op lymfocyten) resulteert in T-cel proliferatie. Na binding van het cytokine op zijn receptor wordt het complex gedeeltelijk geïnternaliseerd. Het extracellulaire gedeelte van de receptor wordt door proteolyse afgesplitst van de membraan en kan in het perifere bloed als oplosbare (soluble) receptor (sIL-2R, sTNF-R1 en sTNF-R2)

aangetoond worden. Wanneer er sprake is van een verminderde afweer in een patiënt, dan kan de afweerreaktie, ofwel immuunresponse op verschillende niveaus verstoord zijn.
In dit proefschrift hebben we getracht een inzicht te krijgen op welk niveau de immuunresponse in patiënten met orgaanfalen en na orgaantransplantatie verstoord is. We hebben ons voornamelijk gericht op de interaktie van de immuuncompetente cellen, de lymfocyten en monocyten, en de centrale cytokinen, TNF-α en IL-2, in patiënten met nierfalen (ESRF), patiënten op hemodialyse (HD) en peritoneale dialyse (CAPD), na niertransplantatie (NT) en bij patiënten met hartfalen (HF) en na harttransplantatie (HTx).

Hoofdstuk 2 beschrijft een verstoorde balans in het TNF-α systeem in patiënten met progressief nierfalen. Dit is het gevolg van sterk verhoogde spiegels sTNF-receptoren. HD patiënten hebben tevens een significant hogere expressie van het mRNA voor TNF-α in tegenstelling tot patiënten op CAPD of met ESRF. De sTNF-R zijn biologisch actief en blijken in staat om 75% van toegevoegd rhTNF-α binden, waardoor de beschikbaarheid van lokaal geproduceerd TNF-α geremd wordt. Hierin ligt mogelijk een verklaring voor de verstoorde immuniteit van HD patiënten

In **hoofdstuk 3** wordt de activatie van het TNF-α systeem op celniveau in HD patiënten aangetoond. Met behulp van de reproduceerbare kwantitatieve flowcytometrische analyse methode hebben we de aanwezigheid van TNF-R2 op lymfocyten en monocyten bepaald. Er is een sterk verhoogde expressie aanwezig van de TNF-R2 op beide celtypen, suggestief voor activatie van het TNF-α. Dit resulteert niet in activatie van het IL-2 systeem, want de expressie van IL-2R α- en β-ketens op de lymfocyt zijn vergelijkbaar met de expressie die gezien wordt op lymfocyten van gezonde controles. Hieruit concluderen we dat de interactie tussen de APC (monocyt) en de lymfocyt verstoord is, daar een duidelijk geactiveerde monocyt, met verhoogde TNF-α mRNA en receptorexpressie, geen activatie van de lymfocyt geeft. Het niveau van TNF-R2 expressie is niet gecorreleerd aan nierfunktie, zodat uremie op zichzelf niet leidt tot de verhoogde cytokinen membraanreceptor expressie.

In **hoofdstuk 4** worden *in vitro* stimulatie testen beschreven van uremische T-cellen, verkregen van HD patiënten teneinde een mogelijk T-cel defect op te sporen. IL-2 mRNA expressie van deze cellen en plasma eiwit spiegels zijn niet verhoogd, maar ook

hier is er sprake van sterk verhoogde concentraties, biologisch actieve sIL-2R. Dit laatste is het gevolg van verminderde renale klaring, welke tot gevolg heeft dat lokaal geproduceerd IL-2 minder beschikbaar is. De mitogeen-geïnduceerde T-cel proliferatie is vergelijkbaar in patiënten en controles, echter alleen als er gecorrigeerd wordt voor de aanwezige hemodialyse-gerelateerde lymfopenie. Uremisch plasma toont een remmend effect op "gezonde" T-cel proliferatie. Tevens is een significant lagere, mitogeen-geïnduceerde TNF-α productie gevonden door T-cellen van HD-patiënten. De conclusie uit deze studie is dat door de continue stimulatie er een uitputting voor TNF-α productie is opgetreden in patiënten op chronische hemodialyse, zonder duidelijk T-cel defect.

In **hoofdstuk 5** wordt de cytokinen afhankelijke T-cel proliferatie verder uitgewerkt. T-cellen van HD patiënten zijn *in vitro* gestimuleerd met TNF-α, IL-2 of IL-15. Dat laatste is een bekende T-cel groeifactor, die geproduceerd wordt door geactiveerde macrophagen. Er is geen verschil in proliferatie capaciteit van T-cellen van HD patiënten en gezonde controles. Stimulatie met combinaties van cytokines, zoals TNF-α met IL2 of TNF-α met IL-15 geeft een verdere toename van de proliferatie capaciteit in beide groepen. Hieruit concluderen wij dat patiënten op HD een goede T-cel functie hebben.

Hoofdstuk 6 toont het beloop van TNF-α en zijn receptoren na een geslaagde living-niertransplantatie. Dagelijks na de niertransplantatie zijn TNF-α mRNA, sTNF-R in bloed en urine gemeten. Bij herstel van de nierfunktie vindt slechts een gedeeltelijk herstel van de TNF-balans plaats. De sTNF-R in het bloed dalen sterk en verschijnen in de urine, echter de plasmaconcentratie van de sTNF-R blijft verhoogd in vergelijking met gezonde controles. Ook na 1 jaar, ongeacht de immuunsuppressieve therapie, cyclosporine of azathioprine, blijven de plasmaconcentraties van sTNF-R verhoogd, resulterend in het blijvend niet in balans zijn van het TNF-α systeem.

In **hoofdstuk 7** is het TNF-α systeem onderzocht in patiënten met ernstig hartfalen (HF) en na harttransplantatie (HTx). TNF-α is behalve om zijn pro-inflammatoire, ook berucht om zijn cardiodepressieve eigenschappen. Er is geen verschil in mRNA expressie voor TNF-α in perifere bloedcellen, echter in patiënten met hartfalen zijn significant hogere TNF-α concentraties in vergelijking met HTx patiënten en gezonde controles gevonden. De sTNF-R zijn in de patiëntengroepen significant hoger dan in de

controlegroep, en ook hier blijken de sTNF-R biologisch actief en zijn in staat om 21% (in HF) en 25% (in HTx) van het toegevoegde TNF-α te binden. In verband met de normale nierfunktie is geen sprake van hoge receptor concentraties door verminderde klaring, maar door overmatige productie. Als de sTNF-R een uiting zijn van een actief TNF-α systeem, dan vindt de activatie niet plaats in de periferie, maar eerder in het falend of in het getransplanteerde hart.

Hoofdstuk 8 beschrijft de activatie van het TNF-α en IL-2 systeem na harttransplantatie op celniveau, gemeten met de kwantitatieve flowcytometrie. Het blijkt dat het TNF-α systeem geactiveerd is, gezien de significant toegenomen TNF-R2 expressie in monocyten en lymfocyten. In tegenstelling tot het TNF-α systeem lijkt het IL-2 systeem juist niet geactiveerd. De expressie van de IL-2R op lymfocyten, zowel de IL-2Rα als de IL-2Rβ keten, is vergelijkbaar in HTx patiënten en gezonde controles. Dit steunt de hypothese dat het geactiveerde TNF-α systeem niet in staat is om de de T-cellen te activeren. In het getransplanteerde hart is een vrijwel continue mRNA expressie voor TNF-α aanwezig. Deze continue TNF-α productie resulteert in op-regulatie van de TNF-R, die na binding met TNF-α leiden tot productie van biologisch actieve sTNF-R. Hierdoor is er ook na HTx sprake van dat het TNF-α systeem niet in balans is, hetgeen resulteert in verdere immuunsuppressie in deze patiënten.

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Curriculum Vitae

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In juli 1996 ben ik gestart met het in dit proefschrift beschreven promotie-onderzoek naar de rol van cytokinen, en met name van TNF-α, in patiënten met orgaanfalen en na transplantatie, onder begeleiding van professor dr. W. Weimar en dr. C.C. Baan, afdeling Inwendige Geneeskunde, AZR-Dijkzigt.
Ik ben sedert 10 maart 1987 getrouwd met Nico van Riemsdijk.

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