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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 cytokine systems with some unbalance, 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 bioavailibility 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, 14 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 infection (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 intrinsic disturbed immune system has to be elucidated. Cytokines play an important part in the regulation of the host defence 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 defence mechanisms against infections or malignant diseases. In a previous study in pe-

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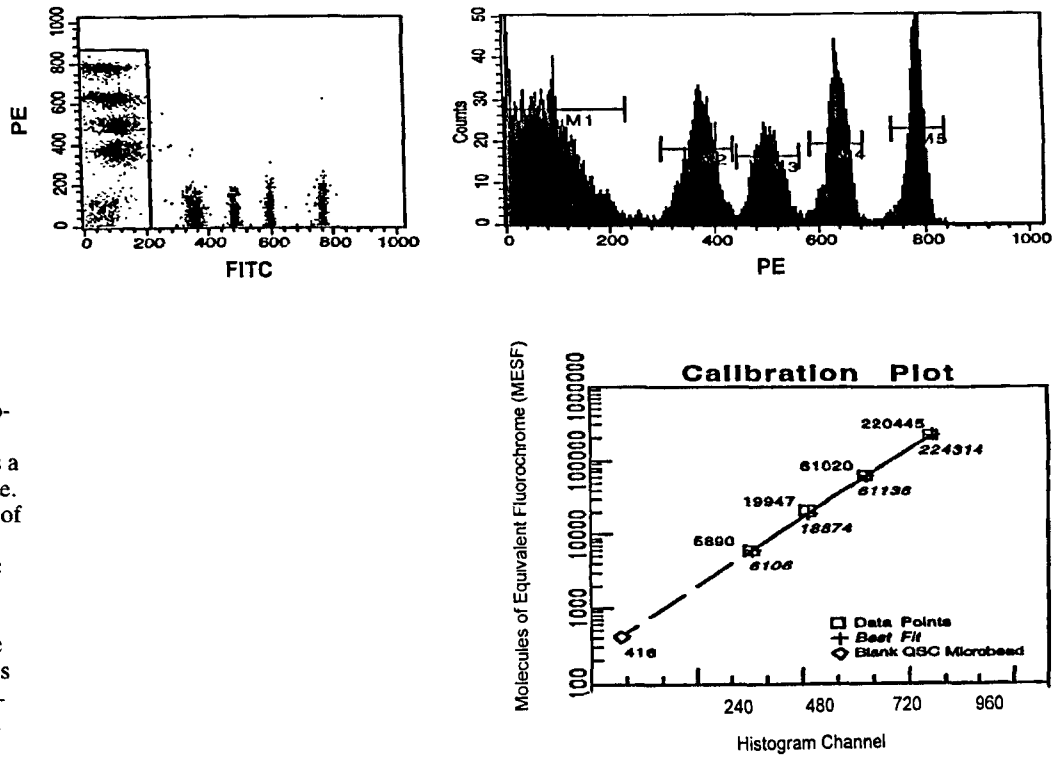

Fig. 1 *Upper panel*: Flow cytometric analysis of calibration beads, each of which contains a specific amount of fluorescene. *Lower panel*: Standard curve of fluorescence intensity constructed after flow cytometric analysis of calibration beads, using Quick Cal program for quantum beads software. The intensity of the fluorescence is depicted as molecular equivalents of soluble fluorochrome (MESF)

ripheral 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 analysed the expression of the TNF-α receptor2 (CD120b) and the IL-2 receptors (CD25 and CD122) on T cells and monocytes 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 (range 43–67) years, 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, infection or malignancy and received cyclosporine and prednisolone 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 analysed 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, Calif.), 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, Paisely, UK) with 0.1% bovine serum albumin (BSA, Sigma, St. Louis, Mo.) 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). 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 (Fig. 1).

Statistical 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.

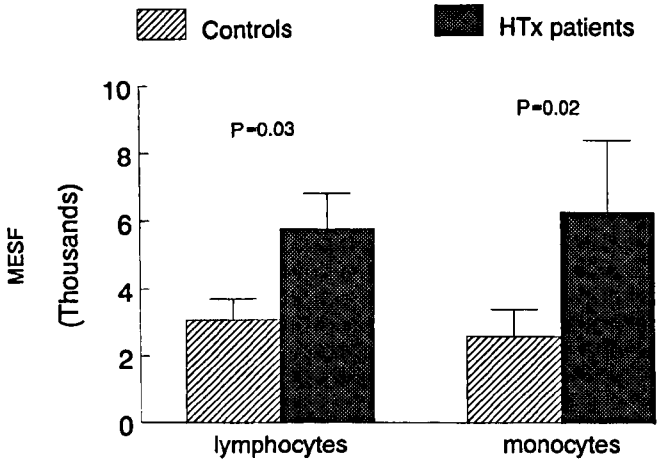

Fig. 2 Expression of the TNF receptor 2 (TNF-R2 = CD120b) on lymphocytes (*left*) and monocytes (*right*) in patients after heart transplantation (*HTx*) compared with controls, using quantitative flow cytometry. The fluorescence is denoted as molecular equivalents of soluble fluorochrome (*MESF*)

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 (234–735 cells/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-(system, TNF-R2 (CD120b) was significantly higher on both TCR$\alpha\beta$ 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 ($P = 0.023$, Fig. 2).

Discussion

After HTx, 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 receptors 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 HTx [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 up-regulation 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 HTx. 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 because of to the high levels of soluble receptors of these cytokines. Previously, we showed that high sTNF-R levels impair the bioavailibility of TNF-α [15]. This, consequently, may influence T cell activation and result in a decreased defence mechanism against infections and malignancy. We postulate that the transplanted heart, as a 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 suppression after heart transplantation.

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