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TNFα in patients with end-stage heart failure on medical therapy or supported by a left ventricular assist device

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Abstract

Background: In the heart elevated levels of TNFα can cause lethal heart failure, like Dilated Cardiomyopathy (DCM). The level of TNFα production is in part determined by promoter gene polymorphisms. We investigated whether the TNFα promoter gene polymorphism is in this way involved in the outcome of end-stage heart failure and predicts whether patients require left ventricular assist device (LVAD) support or can be kept on medical therapy (MT)while awaiting heart transplantation (HTx). As most patients in this study received a heart transplant, the role of the TNFα polymorphisms in transplant rejection was studied as well.
Methods and results: In twenty nine patients with DCM, 35 patients with Ischemic Heart Disease (IHD; both on MT), 26 patients on LVAD support and 61 cardiac transplant donors TNFα plasma level was detected by EASIA. In both patients groups high levels of TNFα plasma levels was observed however, in patients supported by LVAD this increase was much higher compared to patients on MT. Furthermore, this increase seems to be associated with the TNF 1 allele ('G' at position −308) instead of the TNF2 allele (A at position −308). The promoter polymorphisms at positions −238, −244 and −308 were observed by polymerase chain reaction and sequencing. Polymorphism at positions −238, −244 and −308 did not show any relevant differences between the groups. However, at position −308, a trend of a higher incidence of the TNF2 allele (an "A" at position −308) in DCM patients compared to donors was shown. The distribution of the TNF1 and TNF2 alleles was not different in patients on medical therapy compared to the patients supported by a LVAD. No association was found between patients' TNFα promoter gene polymorphism and rejection. However, patients that received a donor heart with the TNF2 allele developed more rejection episodes, compared to patients that received a donor heart with the TNF1 allele.
Conclusion: TNFα levels are high in patients with end-stage heart failure on MT, but even higher in patients on LVAD support. These high TNFα plasma levels however, are not correlated with the TNF2 allele but seems to be associated with the TNF1 allele. Furthermore, in HTx the donor TNFα gene seem to play a more important role in severity of acute rejection than that of the patient.

Keywords: TNFα; LVAD; Promoter gene polymorphism; Heart transplantation

1. Introduction

Cardiovascular disease is the most common cause of death in industrialised nations. Although the death rate from myocardial infarction has declined, the incidence of end-stage heart failure has increased [1]. Beside Ischemic heart disease (IHD), dilated cardiomyopathy (DCM) is a major cause of end-stage heart failure, which is characterised by ventricular dilatation and systolic contractile dysfunction. Heart transplantation (HTx) has

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proven to be an effective long-term treatment for these patients. However, due to the shortage of donor organs some patients with severe end-stage heart failure need to be supported with a left ventricular assist device (LVAD) as bridge to HTx [2–4].

Proinflammatory cytokines are up-regulated in patients with heart failure and have been implicated in the pathophysiology of this disease [5,6]. TNFα, a proinflammatory cytokine, is produced by monocytes and macrophages but also by activated T-cells, fibroblasts, mast cells and NK cells in response to inflammatory or infectious stimuli [7]. Even cardiomyocytes in the failing human heart produce TNFα [8–10]. Low physiologic levels of TNFα confer a cytoprotective effect in the heart [11,12], whereas high levels of TNFα modulate cardiovascular functions through a variety of mechanisms, such as inducing left ventricular dysfunction [13], left ventricular remodelling [14], abnormalities in myocardial metabolism [15], and cardiomyocyte apoptosis [16]. Transcription of TNFα is regulated by transcription factors that bind to transcription sites in the promoter region. Nucleotide variability (polymorphisms) in this promoter region may influence this transcription, and as a consequence the level of TNFα production. In the TNFα gene a lot of different polymorphisms and microsatellites are known, but at least three of them are located in the promoter region and do influence the production of TNFα. In all three polymorphic sites the common nucleotide "G" is substituted by an "A" [17–19]. Wilson et al. described that the "A" at position −308 (indicated as TNF2 allele) constitutes a much more powerful transcriptional site than the "G" at position −308 (indicated as TNF1 allele). Although, no nuclear protein that binds to the polymorphic site is known, they suggested that the polymorphism has a direct effect on the transcriptional activity. They demonstrated a six to sevenfold higher level of transcription from the TNF2 allele in *in vitro* experiments [20]. As different studies [7,9] have shown that TNFα plays an important role in the pathogenesis of end-stage heart failure, we hypothesized that the promoter polymorphism in the TNFα gene influences the development of end-stage heart failure. Because LVAD support can help the patients with the most severe heart problems that would otherwise die on medical therapy awaiting HTx, we investigated whether patients supported by a LVAD carry more often alleles associated with a high TNFα production, than patients on medical therapy. As all patients in our study population received a heart transplantation, we also investigated the influence of the TNFα promoter gene polymorphisms on graft rejection.

2. Materials and methods

2.1. Patient population

The effect of TNFα polymorphisms on heart failure in 29 patients suffering from end-stage DCM (mean age: 45±12) and 35 patients with IHD (mean age: 51±8) both medical therapy prior to heart transplantation (HTx), were compared with twenty-six patients (15 with IHD, mean age: 45±9; 11 with DCM, mean age 33±14) which were supported with a pneumatic or electric LVAD (Heartmate, Thoratec, Pleasanton, California) as bridge to HTx. All patients received a heart transplantation (HTx) between 1999 and 2004.

Sixty one HTx donors were used as control group. All patients were on ACE inhibitor treatment in the stable phase of heart failure. At the time of LVAD implantation, all patients were in NYHA functional class IV, and in NYHA functional class I while on LVAD support. Before LVAD implantation the ACE inhibitor treatment was stopped and all patients received intravenous inotropics because of hemodynamic deterioration. Cardiac medication was discontinued initially in all patients after LVAD implantation. However, in 30% of patients at some stage hypertension was treated by ACE inhibitors again. All of the patients, after HTx were treated with Cyclosporine A, Azathioprine, and Prednisolone triple therapy [21]. Informed consent to participate in this study was obtained from all patients.

2.2. Rejection scores of the patients

Rejection episodes were diagnosed by histopathological examination of endomyocardial biopsies, according to the criteria of the International Society for Heart and Lung Transplantation [22]. HTx patients in this study with a grade 3A or higher were defined as rejectors. Patients with grade 0, 1 and 2 were defined as non-rejectors.

2.3. TNFα plasma concentrations

TNFα plasma levels were detected with EASIA (Biosource Europe S.A., Nivelles, Belgium) following the manufacturers' instructions. Plasma levels were measured pre-HTx or pre-LVAD.

2.4. DNA isolation of the patients and donors

DNA of the patients was obtained from peripheral blood lymphocytes (PBL). DNA of donors was isolated from mononuclear cells from spleen, isolated by Ficoll density gradient centrifugation. DNA was isolated according the salting-out procedure [23].

2.5. Identification of the TNFα gene polymorphism and genotyping

Polymorphisms at positions −238, −244 and −308 in the TNFα promoter region were identified by Polymerase Chain Reaction (PCR) and subsequent sequencing. PCR was performed using the upstream primer, 5′gaaacagaccacagacctg3′ and the downstream primer, 5′ctcacactccccatcc3′, that were both selected with the computer program Oligo (National Biosciences Inc., Plymouth, USA). The PCR mixture contained 500 ng DNA, 1× PCR buffer (Perkin Elmer, Roche Molecular Systems, Inc., Branchburg, New Jersey), 2 mM $MgCl_2$, 0.8 mM dNTPs (Promega Corp., Madison, Wisconsin), 10 pmol upstream primer, 10 pmol downstream primer and 0.75 U AmpliTaq DNA Polymerase (Perkin Elmer) and finally overloaded with mineral oil. Cycling was performed at 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 2 min, with a final cycle of 72 °C for 5 min in a ABI-thermocycler 480 (Perkin Elmer, Foster City, CA). PCR products were purified prior to sequencing to inactivate unincorporated PCR primers and deoxynucleotide triphosphates by enzymatic treatment. This was accomplished by mixing 6 μl exonuclease 1 (10 U/μl; Amersham, Buckinghamshire, UK) and 1 μl shrimp alkaline phosphatase (2 U/μl; Amersham) and incubating at 37 °C for 30 min followed by 80 °C for 20 min. Sequencing was performed by using dye terminator cycle sequencing ready reaction kit (Perkin Elmer). Analysis was done by the computer program ABI Prism™ Sequencing 2.1.1.

2.6. Statistical analysis

All data were calculated using the paired Wilcoxon signed rank test or the Mann Whitney test of the statistical package of Prism 3.02 for Windows. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. TNFα plasma levels in patients on MT and in patients on LVAD support

TNFα plasma levels were measured in patients on MT ($n=64$) and in patients on LVAD support ($n=26$). Patients with end-stage HF either

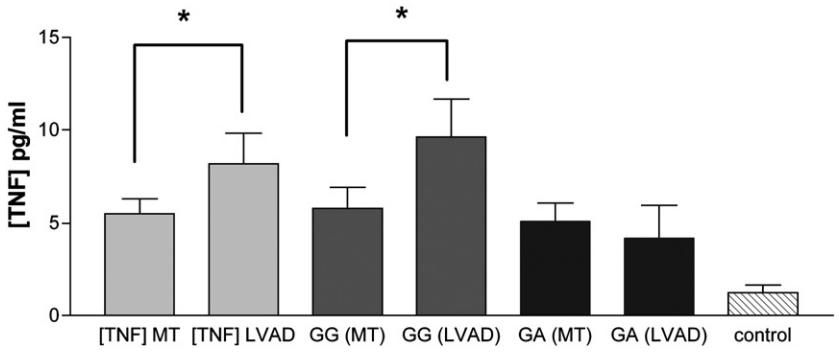

Fig. 1. TNFα plasma levels in patients on medical therapy or LVAD support prior to heart transplantation and their promoter polymorphism on position −308. TNFα plasma levels were significantly increased both in patients on medical therapy (MT) and on LVAD support compared to healthy controls. This increase was even higher in patients with the G polymorphism. *$p<0.05$.

on MT or LVAD support showed significantly increased TNFα levels compared to the healthy controls (Fig. 1; $p=<0.001$). In patients supported with a LVAD this increase is much higher compared to the patients on MT ($p=0.05$) and this increase was augmented in patients which expressed the GG genotype of the TNFα promoter polymorphism at position −308. No significant differences in TNFα plasma concentration was observed in patients expressing the GA genotype, either on MT or LVAD support compared to the control or between the 2 groups of patients.

3.2. TNFα promoter polymorphism in heart failure patients

As shown in Table 1, 29 patients with DCM on medical therapy (MT), 35 patients with IHD on MT, 26 patients on LVAD support and 61 donors were screened for their TNFα promoter polymorphism at positions −238, −244, −308.

The A polymorphism at position −238 was found in 1/29 (3%) patients with DCM on MT, 2/35 (6%) patients with IHD on MT and in 7/61 (12%) of the donors. These differences between the patient and donor groups were not significant. In the patients on LVAD support the A polymorphism at position −238 was found in 2/11 (18%) of the patients with DCM and in 0/15 (0%) of the patients with IHD.

The A polymorphism at position −308 (TNF2) was found in 13/29 (45%) of patients with DCM on MT, in 10/35 (29%) of patients with IHD on MT and in 17/61 (28%) of donors. In patients with DCM a higher frequency of the TNF2 allele was observed compared to IHD and donors. This trend, however was not significant ($p=0.15$). In patients on LVAD TNF2 was found in 4/11 (36%) of the patients with DCM and in 1/15 (7%) of the patients with IHD.

No significant differences were found in the TNFα polymorphism distribution between patients on MT and patients on LVAD support.

The A polymorphism at position −244 was not found in any of the patients or donors (data not shown). This substitution of a “G” to “A” at position −244 was observed almost exclusively in North American Blacks (23). Our patient and donor groups consisted only of Caucasians.

3.3. Association of TNFα promoter polymorphism with heart transplant rejection

Most patients included in this study underwent a heart transplantation.

Only three patients and 7 donors have the A polymorphism at position −238. A correlation could therefore not statistically be defined. Therefore, we correlated the rejectors and non-rejectors during the first six months post heart transplantation with the polymorphism at position −308. There was no significant difference between the TNF1 and TNF2 allele in the rejectors and non-rejectors. However, as shown in Fig. 2, patients who received a donor heart with the TNF2 allele, where more often defined as rejectors, compared to those which received a donor heart with the TNF1 allele ($p=0.03$).

Table 1
TNFα promoter polymorphism at positions −238 and −308 in patients and donors

Heart failure	Position −238			Position −308		
	GG	GA	AA	GG	GA	AA
DCM (MT)	28 (97%)	1 (3%)	0	16 (55%)	12 (41%)	1 (3%)
IHD (MT)	33 (94%)	2 (6%)	0	26 (74%)	9 (26%)	1 (3%)
DCM (LVAD)	09 (82%)	2 (18%)	0	07 (64%)	04 (36%)	0
IHD (LVAD)	14 (93%)	1 (6%)	0	14 (93%)	01 (6%)	0
Donor	54 (89%)	7 (12%)	0	44 (72%)	15 (25%)	2 (3%)

The number of patients and donors are given.
Within brackets: the frequency of each genotype at that position in patient and donor group is given.
DCM = dilated cardiomyopathy, IHD = ischemic heart disease, MT = medical therapy, LVAD = left ventricular assist device.

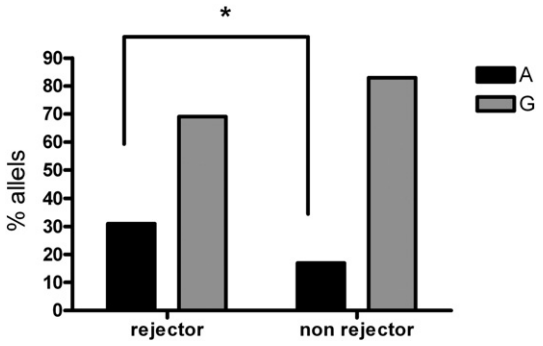

Fig. 2. Frequency of the TNFα promoter polymorphism at position −308 of the donors in relation to rejection. Patients receiving the A polymorphism had more often rejection episodes than patients receiving the G polymorphism. *$p=0.03$. Rejector; ≥ grade 3, non-rejectors < grade 3.

4. Discussion

In this study, we showed that patients with end-stage HF on LVAD support do express significant higher levels of TNFα compared to patients on MT. However, this increased TNFα production is not correlated with the TNF2 polymorphism, but seems to be associated with TNF1. Furthermore we showed that patients that received a donor heart with the TNF2 polymorphism had more severe rejection episodes during the first six months after HTx then patients that received a donor heart with TNF1 polymorphism.

TNFα is produced in the heart by many cells including cardiomyocytes [8–10]. It has been shown that TNFα is up-regulated in the myocardium in response to various forms of cardiac injury, and in addition, plays an important role in the physiology of end-stage heart failure [5]. The use of LVAD in end-stage heart failure has increased over the last 2 decades, since these devices became generally available [24]. In the majority of cases LVAD have been used as a bridge to transplantation. However, in some cases successful explantation of the LVAD was reported [25–29]. Unloading of the heart by a LVAD lead to reversal of LV dilatation, to regression of LV myocyte hypertrophy and to neurohormonal changes [4,30–32]. Patients supported with a LVAD have generally the most severe heart failure and would not survive on medical therapy awaiting HTx. Our hypothesis was that since TNFα plays an important role in the development of end-stage heart failure, that the patients supported by LVAD produce more TNFα and therefore carry more often the TNF2 allele compared to patients that were on medical therapy. In line with our hypothesis, measurement of the TNFα plasma concentration in patients with end-stage heart failure showed that patients who required LVAD support expressed more TNFα protein in their plasma compared to the patients on medical therapy. However, this over expression of TNFα is not linked to TNF2, but seems to be associated with TNF1 expression.

In the present study 29 patients with DCM and 35 patients with IHD which were on medical treatment while waiting for HTx, were screened for their promoter polymorphism and compared to 26 patients with severe end-stage heart failure supported with a LVAD. The TNF2 was found in 45% of the patients with DCM and 29% of the patients with IHD. This indicated a over expression of the TNF2 allele in patients suffering for DCM, but this trend was not significant.

TNF2 was found in 45% of the patients with DCM and 29% of the patients with IHD. This may suggest that TNFα plays a more important role in DCM then IHD. Although there was this trend in the presence of TNF2 in patients suffering for DCM, this was not significant. Densem et al. [28] also studied the TNFα promotor polymorphism in the relation to end-stage heart failure. They did find a significant overrepresentation of the TNF2 allele in the patients with DCM compared to patients with IHD and controls. These data support our data, as in our relative small groups of patients a similar trend was observed.

Interestingly, patients suffering from DCM on LVAD support expressed more often the "A" polymorphism at position −238 compared to patients with DCM on MT (18% compared to 3%). The influence of the polymorphism at the position −238 is not clear yet, but it is possible that this polymorphism can influence the TNFα production as well.

Wilson et al. showed a six to sevenfold higher level of transcription from TNF2 in *in vitro* experiments. It may suggest that the serum levels of TNFα do not correlate with the intra myocardial concentrations of TNFα. TNFα is a proinflammatory cytokine which is released by macrophages and T-cells at the site of inflammation, causing endothelial cell activation, up-regulation of cell adhesion molecules, increasing vasodilatation and local vascular permeability, which will lead to TNFα release in the blood stream. Nevertheless, serum concentrations may not reflect TNFα production in the heart, as other source could be involved as well.

TNFα could also play a role in the severity of rejection after HTx, although conflicting data consist about the role of TNFα and allograft rejection have been described [33–35]. Several studies have suggested that the TNFα polymorphism at position −308 modulate the rejection risk [36,37]. We did not detect a significant difference between the polymorphism in the rejectors and non-rejectors. However, we did show that patients that received a donor heart with TNF2 had more severe rejection episodes during the first six months after HTx than patients that received a donor heart with TNF1. Also the group of Hutchinson et al. has described that the genotype of the donor is important in chronic rejection [38]. Most studies that did report an association between the rejection episodes and the TNFα polymorphism reported a polygenic risk assessment with an IL10 polymorphism [36,37,39]. However, Bedi et al. [40] did not detect any association with the TNF2 polymorphism and an increased risk of allograft rejection. Comparing the various studies on HTx and TNFα polymorphisms it has to be realized that there is a large variation in approach. 1) the complications are not consistent. 2) Some groups define acute rejection as ISHLT grading ≥ 2, while others as ≥ 3A. 3) Non-rejectors were defined as no acute rejection while others included patients with grading 1 and 2. 4) Time in which the rejection episodes occur was different between studies, and 5) Differences in immunosuppressive regimes [41].

Taken all data together this study showed that TNFα is an important factor in the development of heart failure and in line with our hypothesis in patients who required LVAD support, higher TNFα plasma levels were detected compared to patients on MT and healthy controls. However, this TNFα production is not associated with the TNF2 allele, but seems to correlate with the TNF1 allele. Furthermore, the TNFα polymorphism of the donor seems to play an important role in transplant rejection. Unfortunately, our data on TNFα gene polymorphism however do not reach significance. A large international multi center study is necessary to increase the patient group to see whether the trend become significant. Furthermore, TNFα is a complicated cytokine which acts not on its own but is part of a multiplex of factors including other cytokines and growth factors.

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