Tumor Necrosis Factor-α Is Expressed in Donor Heart and Predicts Right Ventricular Failure After Human Heart Transplantation

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**Background**—Myocardial failure is an important problem after heart transplantation. Right ventricular (RV) failure is most common, although its mechanisms remain poorly understood. Inflammatory cytokines play an important role in heart failure. We studied the expression of tumor necrosis factor (TNF)-α and other cytokines in donor myocardium and their relationship to the subsequent development of RV failure early after transplantation.

**Methods and Results**—Clinical details were obtained, and ventricular function was assessed by transesophageal echocardiography in 26 donors before heart retrieval. A donor RV biopsy was obtained immediately before transplantation, and each recipient was followed for the development of RV failure. Reverse transcriptase–polymerase chain reaction was performed to detect TNF-α, interleukin-2, interferon-γ, and inducible nitric oxide synthase expression. Eight of 26 recipients (30.8%) developed RV failure. Seven of these 8 (87.5%) expressed TNF-α, but only 4 of the 18 (22.2%) who did not develop RV failure expressed TNF-α ($P<0.005$). As a predictor of RV failure, TNF-α mRNA had a sensitivity of 87.5%, a specificity of 83.3%, a positive predictive value of 70%, and a negative predictive value of 93.7%. Western blotting demonstrated more TNF-α protein in the myocardium of donor hearts that developed RV failure (658±66 versus 470±57 optical density units, $P<0.05$). Immunocytochemistry localized TNF-α expression to cardiac myocytes. Reverse transcriptase–polymerase chain reaction detected interferon-γ in 2 (7.7%), interleukin-2 in 1 (3.8%), and inducible nitric oxide synthase mRNA in 1 (3.8%) of the 26 donor hearts, none of which developed RV failure.

**Conclusions**—TNF-α expression in donor heart cardiac myocytes seems to predict the development of RV failure in patients early after heart transplantation. (*Circulation*. 2000;102:326-331.)

**Key Words:** transplantation ■ myocardium ■ contractility

A significant number ($\approx20\%$) of cardiac donors are not used for transplantation because of poor donor myocardial function,$^1$ and 30%$^2$ of early deaths after transplantation reported by the Registry of the International Society of Heart and Lung Transplantation occur because of myocardial failure. Right ventricular (RV) dysfunction is most commonly seen$^3$ in the clinical setting; this is the result of either elevated pulmonary vascular resistance in the recipient or donor myocardial dysfunction. Experimental evidence suggests that the right ventricle is more susceptible to ischemia-reperfusion injury than the left ventricle.$^4$ Both RV and left ventricular dysfunction occur after brain death in potential organ donors. Experimental studies have shown more pronounced changes in RV function.$^3$-$^5$-$^7$ The mechanisms producing RV dysfunction remain poorly understood.

Patients with chronic heart failure due to a variety of causes have an elevated expression of the proinflammatory cytokines, including tumor necrosis factor (TNF)-α, both in the serum and myocardium.$^8$-$^15$ TNF-α depresses myocardial contractile function,$^15$-$^17$ either directly or through the induction of inducible nitric oxide synthase (iNOS). The proinflammatory cytokines interleukin (IL)-2 and interferon-γ (IFN-γ) can induce TNF-α production.$^18$

Therefore, we investigated the relationship between the myocardial expression of TNF-α and other cytokines in the myocardium of donor hearts and the development of right heart failure early after transplantation, as well as the possible mechanisms of its induction and/or action in this setting.

**Methods**

**Donors**

Donor details are shown in the Table. Ten were domino hearts (from patients undergoing heart-lung transplantation) and 16 were hearts...
from brain-dead donors. Causes of death were subarachnoid hemorrhage (n=8), intracranial infarct (n=3), head injury (n=2), subdural hemorrhage (n=1), asthma (n=1), and meningitis (n=1). Infection in both the living and brain-dead donors was defined as a raised white cell count combined with either pyrexia or evidence of consolidation on a chest x-ray. The protocol was approved by the Hillingdon Health Authority Ethics Committee. Transesophageal echocardiograms at the time of retrieval showed that all the donors had an ejection fraction >55%. Mean central venous pressure was 8.9 ±1.0 mm Hg (range, 4.0 to 16.0 mm Hg). Preservation of the donor heart was done by the infusion of St Thomas’ solution at 4°C. Mean ischemia time was 134 ±11 minutes (range, 35 to 210 minutes). RV endomyocardial biopsies were obtained from the donor heart immediately before implantation and frozen in liquid nitrogen for analyses with reverse transcriptase–polymerase chain reaction (RT-PCR) and Western blotting. A further biopsy was fixed in 10% formal saline for immunocytochemistry.

Recipient Details

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<td>Period of ventilation, days, mean (range)</td>
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Recipient Details

The recipients of the donor hearts included 21 men and 5 women with a mean age 46.8 years (range, 27 to 62 years). Indications for transplantation were dilated cardiomyopathy (n=13), ischemic heart disease (n=11), postpartum cardiomyopathy (n=1), and myocarditis (n=1). New York Heart Association class before transplantation was III in 19 recipients and IV in 7 recipients. Before transplantation, mean pulmonary artery pressure was 29.2 ±2.2 mm Hg, mean pulmonary capillary wedge pressure was 20.7 ±2.2 mm Hg, mean transmural gradient was 8.3 ±1.1 mm Hg, and pulmonary vascular resistance ranged from 1 to 8 Wood units (mean, 3.5 ±0.6 Wood units). Development of right heart failure was defined as a dilated, poorly contracting right ventricle observed by transesophageal echocardiography in the presence of low cardiac output syndrome (defined as ≥2 of the following: urine output <0.5 mL · kg⁻¹ · h⁻¹; inotrope [epinephrine or norepinephrine] requirements of >0.5 μg · kg⁻¹ · min⁻¹ necessary to maintain a systolic blood pressure >90 mm Hg; cardiac index <2; and progressive metabolic acidosis) and in the presence of adequate left atrial filling pressures.

RT-PCR

RT-PCR was performed for TNF-α, IL-2, IFN-γ, and iNOS on all donor biopsies.

RNA Extraction and Preparation of cDNA

Total RNA was extracted from donor RV biopsies as described previously. cDNA was prepared by reverse transcription of 500 ng of total RNA with Moloney murine leukemia virus RT (Rnase reverse transcriptase) enzyme using oligo (dT)₁₂₋₁₈ as a primer. After transcription at 37°C for 60 minutes, the reaction mixture was denatured at 95°C for 5 minutes, chilled on ice, and stored at −20°C until required for PCR amplification.

PCR Amplification

cDNA was amplified in a PCR reaction containing 20 μmol/L each deoxynucleotide triphosphate, 20 pmol/L each primer, 50 μmol/L KCl, 10 mmol/L Tris HCl (pH 8.4), 0.1 mg/ml gelatin (Sigma), and 1 to 1.5 mmol/L MgCl₂. A total of 45 amplification cycles with denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C were performed. Products were analyzed by ethidium bromide staining after gel electrophoresis.

Oligonucleotide primers to TNF-α, iNOS, and GAPDH were designed in-house from published sequences. Sequences for TNF-α were as follows: sense: CACACGCCTCTTCTGCTGGC (HUMTNFAA 217 to 236) and antisense: TCTCGTCAACGC- CATT (HUMTNFAA 445 to 426). iNOS and GAPDH primers have been described previously; they were as follows: iNOS sense ATTGATGCAAGCTGTCC (HUMINOSA 2127 to 2145), antisense: GTAGATCTCGCCAGATTTG (HUMINOS 2392 to 2411), GAPDH sense: TCACATCTTCCAGGAGCCA (HSGBPDR 281 to 300), and antisense: TCTTGAGGCACTGTGGCGC (HS- GAPDR 1045 to 1064). IFN-γ primers were described previously, and IL-2 primers were commercially available (Clontech Laboratories, Inc). All primers were intron-spanning.

Western Blot Analysis

Total protein extracts were prepared from 18 of the donor biopsies (in which sufficient tissue was available), 7 of which developed RV failure, by homogenising myocardial biopsies in lysis buffer (1% SDS, 1 mmol/L phenylmethylsulfonylfluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Protein (40 μg per sample) was loaded onto a 12.5% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred onto Hybond-C super nitrocellulose membranes. Membranes were immersed in PBS-Tween 20 and 5% milk protein overnight at 4°C to block nonspecific binding. Primary TNF-α (Santa-Cruz Biotechnology), diluted 1:500 (v/v) in PBS-Tween 20/5% milk protein, was added to the membranes for 60 minutes. After washing, membranes were subsequently incubated with a horseradish-peroxidase–conjugated rabbit anti-goat secondary antibody (DAKO) diluted 1:1000 (v/v) in PBS-Tween 20/5% milk protein for 1 hour. The immunoreactive bands were visualized using Amersham electrogenerated chemiluminescent reagents, and they were scanned using Image Analysis 1000 software (Alpha Innotech).

Immunocytochemistry

Immunocytochemistry was performed on formalin-fixed paraffin-embedded sections from 17 donor hearts (in which sufficient tissue was available), 6 of which developed RV failure, to localize the cell type producing TNF-α. The avidin-biotin-peroxidase complex method was used. Endogenous peroxidase was blocked with 0.03% (v/v) hydrogen peroxide in methanol for 20 minutes. After incubation with normal goat serum (1:30, 30 minutes), sections were incubated overnight at 4°C with primary rabbit antibodies to TNF-α (Antigenex America Inc), diluted 1:100 (v/v). Immunoreaction sites were visualized using the appropriate biotinylated secondary antibody and the avidin-biotin-peroxidase complex procedure (Vector Labs). Peroxidase activity was revealed with a solution of diaminobenzidine as chromogen with 0.2% (v/v) hydrogen peroxide in PBS to produce a brown reaction product; sections were counterstained with Harris’ hematoxylin. Controls consisted of the replacement of primary antibodies with nonimmune rabbit serum. Semiquantitative analysis was performed using a BH-60 microscope (Olympus). The degree of TNF-α expression was assessed by 2 independent observers and graded on a scale from 1 to 3 for each cell type in each biopsy.

Statistics

Variables are expressed as mean ± SEM. Fisher’s exact test using a 2×2 contingency table and Student’s t test were used for the analysis. The sensitivity, specificity, positive predictive values, and negative predictive values were calculated using standard formulae. P <0.05 was considered significant.

Results

Heart Failure and Patient Outcome

Eight of the 26 patients (30.8%) developed right heart failure after transplantation. These patients had a significantly longer
monary gradient. The other 7 domino hearts from patients with cystic fibrosis did not express TNF-α and did not develop RV failure when transplanted into patients with low transpulmonary gradients. All patients with cystic fibrosis had extensive chronic inflammatory changes with a large amount of purulent secretions in both lungs. In addition, 2 patients had evidence of active infection (pyrexia with growth of *Proteus* species in one and pyrexia in the other) at the time their heart was used for transplantation, but in neither heart was TNF-α expressed.

TNF-α expression was not affected by donor age, central venous pressure, period of ventilation, sex, smoking status, use of inotropes, or ischemia time (*P*=NS). TNF-α was not affected by the cause of death (TNF-α was positive in 3 of the 8 donors with subarachnoid hemorrhage, 2 of the 3 with intracranial infarcts, the donor with a head injury, and the donors with subdural hemorrhage and asthma; it was not expressed in the donor with meningitis). TNF-α was not affected by the presence of donor infection (*P*=NS).

### TNF-α Protein Expression by Western Blotting
Significantly higher TNF-α expression existed in the donor hearts of patients who developed right heart failure (658.9±60 optical density [OD] units, *n*=7) compared with those who did not (470.5±57 OD units, *n*=11; *P*<0.05) (Figure 2). A strong trend existed for higher TNF-α protein expression in the donors expressing TNF-α mRNA (637.3±71 OD units) compared with those who did not (474.3±61 OD units, *P*=0.07).

### TNF-α Expression by Immunocytochemistry
TNF-α expression was localized to cardiac myocytes (Figure 3); it was not expressed in vascular smooth muscle cells and was only occasionally expressed in endothelial cells (only one biopsy) (Figure 3). Semiquantitative analysis showed stronger TNF-α expression in the cardiac myocytes of patients developing right heart failure (score, 1.1±0.4 versus 0.6±0.1 arbitrary units) (Figure 3), although this did not reach statistical significance. A strong trend existed for higher TNF-α protein expression in cardiac myocytes from the donors who expressed TNF-α mRNA (1.1±0.3 versus 0.5±0.1 arbitrary units; *P*=0.09).

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**Figure 1.** Donor heart TNF-α expression and development of RV failure in (a) all donors and (b) brain-dead donors.

**Figure 2.** Densitometric analysis of protein bands from Western blots incubated with TNF-α antibody shows significantly higher TNF-α expression in the myocardium of the group of 7 donor hearts that developed right heart failure (RSHF) compared with the 11 that did not (Non RSHF).

stay in the intensive therapy unit (mean, 4.1±0.7 days) than those who did not develop RV failure (mean, 1.4±0.27 days; *P*<0.001). Two of these 8 patients (25%) required intra-aortic balloon counterpulsation in the postoperative period, compared with none of the 18 who did not develop RV failure. Patients with RV failure spent a mean of 43.5±6 days in the hospital (including readmissions) in the first 3 postoperative months, compared with 30.2±3 days for those who did not develop RV failure (*P*<0.05).

**TNF-α mRNA Expression**

RT-PCR showed that 7 of the 8 patients (87.5%) who developed right heart failure expressed TNF-α mRNA compared with 4 of the 18 patients (22.2%) who did not (*P*<0.005). None of the known risk factors for the development of RV failure (such as donor age, preharvesting presence/absence of inotropes, ischemia time, or recipient transpulmonary gradient) was present in this group of patients. As a predictor of right heart failure, TNF-α had a sensitivity of 87.5%, a specificity of 83.3%, a positive predictive value of 70%, and a negative predictive value of 93.7% (Figure 1a). Of the 16 hearts from brain-dead donors, 7 (43.8%) developed RV failure and 6 of these 7 (85.7%) expressed TNF-α (Figure 1b).

Ten of the 26 donor hearts (38.5%) were domino donor hearts; 9 of these were from cystic fibrosis patients and 1 was from a patient with primary pulmonary hypertension. Two of the 10 domino hearts (20%), both from patients with cystic fibrosis, expressed TNF-α mRNA. One of these 2 hearts developed RV failure after transplantation into a patient with a low transpulmonary gradient. The domino heart from the patient with primary pulmonary hypertension did not express TNF-α when transplanted into a patient with a high transpulmonary gradient.
Other Cytokine Expression

RT-PCR detected IFN-γ in 2 (7.7%), IL-2 in 1 (3.8%), and iNOS mRNA in 1 (3.8%) of the 26 donor hearts, and none of these hearts developed RV failure. Only one of the 2 hearts expressing IFN-γ also expressed TNF-α, and the heart that expressed IL-2 did not express TNF-α mRNA. In the one heart with iNOS expression, no TNF-α expression occurred.

Discussion

This study documented, for the first time, a relationship between both TNF-α mRNA and protein expression in the myocardium of donor hearts and the development of right heart failure early after transplantation. Myocardial failure after transplantation remains an important problem, and it probably has a variety of causes, including the effects of brain death, donor management, and ischemia-reperfusion during harvesting and implantation.

Studies of cardiac function after brain death in experimental animals by Bittner and colleagues have shown that biventricular systolic function and contractility were signifi-
myocardial homogenates (Western blotting) and in myocytes (immunocytochemistry), although this was not statistically significant.

TNF-α is a proinflammatory cytokine\textsuperscript{15} that is released locally in response to infection and injury. Previous studies have shown elevated plasma concentrations of TNF-α in patients with heart failure.\textsuperscript{8–12} Furthermore, levels of TNF-α have been associated with decreasing patient functional status,\textsuperscript{10,11} and a trend has been found between higher TNF-α concentration and impaired survival.\textsuperscript{10} TNF-α expression has been demonstrated\textsuperscript{13,14} in the ventricles of patients with dilated cardiomyopathy, where it was seen in cardiac myocytes, endothelial cells, and in the vascular smooth muscle cells of intramyocardial blood vessels. A recent study\textsuperscript{25} showed that the unloading of the failing human heart by the placement of a left ventricular assist device is associated with a reduction in TNF-α expression. The greatest reduction occurred in patients who recovered cardiac function and were successfully weaned off the device, suggesting that the putative effect of the cytokines on myocardial function could be reversible.

The mechanism of TNF-α induction in our patients remains unknown. No relationship existed between TNF-α induction and infection, period of ventilation, smoking status, use of inotropes in the donor, or ischemia time. Although TNF-α production can be stimulated by pressure overload,\textsuperscript{26} no correlation existed between TNF-α expression and the central venous pressure of the donor before explantation. However, during and immediately after brain death, extensive changes in loading conditions occur. In our study, the correlation between TNF-α mRNA expression and the development of right heart failure in the brain-dead donors suggests that TNF-α expression may result from brain death. Others have found\textsuperscript{27} that after the induction of brain death in the rat, TNF-α mRNA can be detected in the heart and other peripheral organs. In that study, IL-1, IL-2, IL-6, and IFN-γ mRNA were also detected. No relationship existed between TNF-α expression and the cause of brain death in our study. TNF-α is likely to be upregulated very quickly in cardiac myocytes after brain death because it is an “acute phase reactant.” This is analogous to the clinical situation of cardiopulmonary bypass in which TNF expression has also been observed.\textsuperscript{28}

TNF-α production can be induced by the proinflammatory cytokines IL-2 and IFN-γ.\textsuperscript{18} IL-2 was only detected in one donor in our series and that heart did not express TNF-α, and IFN-γ was only detected in 2 donor hearts, one of which expressed TNF-α. This suggests the expression of IL-2 and IFN-γ is not the mechanism through which TNF-α is produced in our patients.

Bozkurt et al\textsuperscript{16} and Yokoyama et al\textsuperscript{17} showed that TNF-α can depress myocardial function. The hemodynamic effects of TNF-α are characterized by decreased myocardial contractile efficiency and reduced ejection fraction, hypotension, and biventricular dilatation.\textsuperscript{15} TNF-α may affect graft myocardial function through NO-dependent or NO-independent mechanisms. TNF-α can induce the expression of iNOS,\textsuperscript{20,29} which can result in the production of large quantities of NO.\textsuperscript{3} NO may have a negative inotropic effect.\textsuperscript{8,30} We found iNOS expression in only one donor heart, and this donor did not express TNF-α; this suggests that in the donor heart, TNF-α is acting through a NO-independent mechanism. TNF-α binds to 2 receptors, TNFR1 and TNFR2. Binding to TNFR1 results in sphingosine production, which decreases calcium transients and may lead to dysfunctional excitation-contraction coupling and to systolic and/or diastolic dysfunction.\textsuperscript{13,31} TNF-α can also induce the apoptosis of cardiac myocytes through a sphingosine-dependant mechanism.\textsuperscript{32} Furthermore, TNF-α causes a concentration-dependant increase in the inhibitory G protein Gi\textsuperscript{33} in rat cardiac myocytes, and our group have previously demonstrated increased activity of Gi\textsuperscript{α} in donor hearts with myocardial dysfunction.\textsuperscript{23} In addition, TNF-α has the potential to lead to heart failure through its effects on matrix metalloproteinases.\textsuperscript{34}

The patients in our series who developed right heart failure had longer stays in the intensive therapy unit and spent more time in the hospital during the first 3 months after transplantation, which suggests that donor myocardial dysfunction affects patient outcome (as others have shown previously\textsuperscript{2,25}) and increases the cost of patient care.

Modulation of TNF-α expression might avoid right heart failure in some recipients and could improve the function of “marginal” donors, which might lead to an expansion of the useable donor pool. Etanercept is a p75 TNF receptor fusion protein that binds to TNF-α, thus functionally inactivating it. When given to patients with New York Heart Association class III heart failure in a randomized double-blind trial, it increased their quality of life, 6-minute walk distances, and ejection fractions.\textsuperscript{36} Pentoxifylline is a xanthine derivative that suppresses or reduces the production of TNF-α\textsuperscript{37} and is thought to act at the mRNA level.\textsuperscript{38} Administration of pentoxifylline to patients with idiopathic dilated cardiomyopathy\textsuperscript{39} resulted in improved functional class, increased ejection fraction, and decreased TNF-α levels. When administered in an animal model\textsuperscript{40} of lung transplantation, pentoxifylline improved oxygen tension, pulmonary vascular resistance, and recipient survival.

**Limitations**

Although we demonstrated the expression of TNF-α in the right ventricle, we have no data relating to its expression in the left ventricle. Although there are plausible biological mechanisms by which TNF-α expression may lead to ventricular dysfunction, our study cannot prove that TNF-α is the only mechanism causing RV failure in these patients.

**Conclusions**

Our results suggest that TNF-α expression in the donor heart is an important predictor of the development of right heart failure early after transplantation and that TNF-α may contribute to myocardial dysfunction. Our results indicate that TNF-α does not act through an NO-dependant mechanism but may act through other pathways. Pharmacological modulation of TNF-α expression in organ donors may be a useful strategy for reducing post-transplant ventricular dysfunction.

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