Quantitative Myocardial Cytokine Expression and Activation of the Apoptotic Pathway in Patients Who Require Left Ventricular Assist Devices

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Background—Molecular mechanisms underlying the deterioration of patients undergoing LV assist device (LVAD) implantation remain poorly understood. We studied the cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-1β and IL-6 and the terminal stage of the apoptotic pathway in patients with decompensating heart failure who required LVAD support and compared them with patients with less severe heart failure undergoing elective heart transplantation.

Methods and Results—Myocardial and serum samples from 23 patients undergoing LVAD implantation were compared with those from 36 patients undergoing elective heart transplantation. Myocardial TNF-α mRNA (1.71-fold; P<0.05) and protein (3.43±0.19 versus 2.95±0.10 pg/mg protein; P<0.05) were elevated in the LVAD patients. Immunocytochemistry demonstrated TNF expression in the myocytes. Serum TNF-α was also elevated (12.5±1.9 versus 4.0±0.4 pg/mL; P<0.0001) in the LVAD patients. IL-6 mRNA (2.57-fold higher; P<0.005) and protein (27.83±9.35 versus 4.26±1.24 pg/mg protein; P<0.001) were higher in the LVAD candidates, as was serum IL-6 (79.3±23.6 versus 7.1±1.6 pg/mL; P<0.0001). Interleukin-1β mRNA expression was 9.78-fold higher in the LVAD patients (P<0.001). iNOS mRNA expression was similar to that in advanced heart failure patients and was not further elevated in the LVAD patients. Levels of procaspase-9 (8.02±0.91 versus 6.16±0.43 oligodeoxynucleotide [OD] units; P<0.01), cleaved caspase-9 (10.02±1.0 versus 7.34±0.40 OD units; P<0.05), intact and spliced DFF-45 (4.58±0.75 versus 2.84±0.23 OD units; P<0.05) were raised in LVAD patients, but caspase-3 and human nuclease CPAN were not.

Conclusions—Elevated TNF-α, IL-1β, and IL-6 and alterations in the apoptotic pathway were found in the myocardium and elevated TNF-α and IL-6 in serum of deteriorating patients who required LVAD support. These occurrences may have therapeutic implications and influence the timing of LVAD insertion. (Circulation. 2001;104[suppl I]:I-233-I-240.)

Key Words: heart-assist device ▪ interleukins ▪ heart failure ▪ nitric oxide synthase

Left ventricular assist devices (LVADs) have become an established treatment for patients with severe heart failure. Molecular mechanisms underlying the decompensation of heart failure remain poorly understood. Understanding the mechanisms involved may help with decisions about timing of LVAD implantation and identifying new therapeutic targets in advanced heart failure.

Expression of the proinflammatory cytokine tumor necrosis factor (TNF)-α has been described in patients with chronic heart failure both in serum and myocardium,1–5 and serum levels have been found to correlate with functional status.2 TNF-α has been shown to produce myocardial depression both in in vitro and in vivo models.2,3,6 Interleukin (IL)-6 also is elevated in myocardium and serum of patients with heart failure, and levels correlate with poor functional status.1,3,7,8 IL-1β is known to cause myocardial depression in vivo9,10 and acts synergistically with TNF-α,10 but its role in heart failure is unclear.

TNF-α and IL-1β can activate inducible nitric oxide synthase (iNOS), and their negative inotropic effect can be mediated through iNOS induction.11 iNOS expression has been described in patients with heart failure,4 iNOS is a potent producer of nitric oxide, which can have a negative inotropic effect. TNF-α and IL-1β also can induce apoptosis of cardiac myocytes,12,13 whereas IL-6 has antiapoptotic effects.14,15

Apoptosis is tightly regulated by the caspases, which initially are translated as inactive proenzymes and are subsequently cleaved to become active. In the end stage of the apoptotic pathway, release of cytochrome c from the mito-
chondron activates procaspase-9 to caspase-9, which, in turn, activates procaspase-3 to caspase-3. Caspase-3 activates DNA fragmentation factor, a heterodimer that consists of the active capase-activated nuclease (CPAN)/DFF-40 complex, a 40-kDa nuclease, and DFF-45, its 45-kDa inhibitor. Caspase-3 cleaves DFF-45 from the CPAN/DFF-40–DFF-45 complex and generates the functionally active CPAN nuclease, which induces chromatin condensation and DNA fragmentation. Poly(ADP-ribose) polymerase (PARP), a DNA repair enzyme, is inactivated by caspase-3, which contributes to the demise of the cell. Our group and others have previously demonstrated evidence of activation of the apoptotic pathway in advanced heart failure.

These mechanisms all could contribute to decompensation of patients with advanced heart failure, who then require LVAD support. To investigate this hypothesis, we have quantified myocardial TNF-α, IL-1β, and IL-6 expression; circulating TNF-α, its receptors, and IL-6; and myocardial expression of iNOS and of caspases in the terminal stage of the apoptotic pathway in patients who require insertion of an LVAD and compared them with patients with stable advanced heart failure awaiting heart transplantation. Our aim was to characterize better patients for which the findings could have implications for their management and to help to understand factors that could influence progression of heart failure.

Methods

Patients

Protocol for the present study was approved by the Royal Brompton and Harefield Research Ethics Committee. Informed consent was obtained from each patient.

LVAD Patients

The present study included 23 consecutive patients who required LVAD implantation because of deteriorating clinical status with cardiac output (cardiac index < 2) or low ventricular function (LVEF < 25%) and the reverse primer was 5′-GGCCAAAGGGATTTTAACTTGCAG-3′. The iNOS probe designed was 5′-CACCATAATGCACTCCAC-3′.

Serum markers were studied in all 23 patients (male, n = 20; female, n = 3; age range, 14 to 58 years; mean age, 37 ± 3.1 years). Patients were diagnosed as having dilated cardiomyopathy (n = 17), ischemic heart disease (IHD; n = 2), IHD with postinfarct ventricular septal defect rupture (n = 2), postpartum cardiomyopathy (n = 1), or congenital heart disease (n = 1). All were patients in New York Heart Association (NYHA) class IV and had deteriorated over a period of 3.2 ± 0.6 years. Mean pulmonary capillary wedge pressure (PCWP) was 26.0 ± 2.1 mm Hg. Mean LV end diastolic diameter (LVEDD) was 73.8 ± 6.0 mm, and LV end-systolic diameter (LVESD) was 64.8 ± 6.3 mm. Blood was collected from each patient immediately before insertion of the device.

Myocardial markers were studied in the first 13 of 23 patients (male, n = 10; female, n = 3; age range, 14 to 58 years; mean age, 35.5 ± 4.1 years). These patients were diagnosed as having dilated cardiomyopathy (n = 11), ischemic heart disease with postinfarct ventricular septal defect rupture (n = 1), or postpartum cardiomyopathy (n = 1). All were in NYHA class IV and had deteriorated over a period of 2.3 ± 0.6 years. Mean PCWP was 27.3 ± 2.5 mm Hg. Mean LVEDD was 75.5 ± 7.9 mm, and LVESD was 64.8 ± 9.8 mm. A core of myocardium from the apex of the LV was taken at the time of LVAD insertion, instantly frozen in liquid nitrogen, and stored at −80°C for subsequent analysis.

Heart Failure Patients

Patients with less-severe heart failure who were undergoing heart transplantation and did not meet our criteria for LVAD implantation acted as controls. Serum markers were studied in 17 patients (men, n = 14; women, n = 3; age range, 22 to 64 years; mean age, 46.1 ± 3.3 years). NYHA class was III in 13 and IV in 4. Patients were diagnosed as having dilated cardiomyopathy (n = 9), ischemic heart disease (n = 7), or postpartum cardiomyopathy (n = 1). Mean PCWP was 25.4 ± 2.2 mm Hg, mean LVEDD was 70 ± 2 mm, and mean LVESD was 61.2 ± 3 mm.

Myocardial markers were studied in 36 patients (male, n = 30; female, n = 6; mean age, 46 ± 3.4 years). NYHA class was III in 31 and IV in 5. Patients were diagnosed as having dilated cardiomyopathy (n = 18), ischemic heart disease (n = 15), postpartum cardiomyopathy (n = 2), or myocarditis (n = 1). Mean PCWP was 21.9 ± 1.5 mm Hg, mean LVEDD was 71.4 ± 2.6 mm, and mean LVESD was 62.6 ± 2.5 mm. A sample of LV near the apex was taken at the time of transplantation, instantly frozen in liquid nitrogen, and stored at −80°C for subsequent analysis.

For all LVAD and heart failure patients, blood was spun within 4 hours of collection at 2500 rpm for 10 minutes, and serum supernatant was stored at −40°C for analysis.

Cytokines

Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction

TNFα, IL-1β, IL-6, and iNOS mRNA was detected by polymerase chain reaction (PCR) amplification, quantified by 5′ nucleic assay with fluorescence-labeled TaqMan probes and analyzed by use of real-time quantitative PCR as follows. Total RNA was extracted by use of Qiagen Inc RNeasy minicolumns and eluted in diethylpyrocarbonate-treated DH2O. RNA quality and quantity was assessed by EtBr-agarose gel electrophoresis and by relative absorbance at 260 versus 280 nm. cDNA was synthesized from 150 ng of total RNA by use of the PE Biosystems reverse-transcriptase kit with random hexamer primers. Reactions were diluted to 100 µL. Primers and TaqMan probes for human IL-6 and iNOS were designed. Primer Express software (PE Biosystems) was used to design the IL-6 probe from a published mRNA sequence (EMBL/GenBank accession No. M54894), which gave an amplicon size of 96 bp with the TaqMan probe straddling the exon-exon junction (forward primer [Tm = 58°C], 5′-TGACAAACAAATTCGGTACATCCT-3′; reverse primer [Tm = 60°C], 5′-AGTGCTCTTGTGCTTCTAC-3′; TaqMan probe [Tm = 68°C] 5′-TTACTCTGTATCATGTCTTCT TCAAGGGCT-3′). The iNOS probe designed was 5′-CACATAAG GGCACAAAGGAGGTATTTAATGCACG-3′ (Tm = 70°C), the forward primer was 5′-AGCGGGATGACTTCTTAA-3′ (Tm = 58°C), and the reverse primer was 5′-TAATGACCCCGAGCAGATTT-3′ (Tm = 59°C).

Perkin-Elmer primers and TaqMan probes for TNF-α and IL-β were used. PCR reactions were performed by use of an ABI-prism 7700 sequence detector. PCR amplifications were performed in a 25-µL volume that contained a 2.5-µL cDNA template in 2X PCR Master Mix (PE Biosystems) at 50°C for 2 minutes and 95°C for 10 minutes followed by 40 cycles of 95°C for 15 s and 60°C for 1 minute. Results were analyzed by use of Sequence Detection Software (PE Biosystems), and the level of expression of TNF-α, IL-1β, IL-6, and iNOS mRNA was normalized to 18S rRNA as outlined in User Bulletin No. 2 provided by Perkin-Elmer.

Myocardial Immunoassay

Protein was extracted from myocardial tissue. Protein preparations were made by homogenizing myocardial biopsies in 20 mMol/L of HEPES and 1.5 mMol/L of EDTA solution that contained protease inhibitors aprotinin, leupeptin, DTT, and phenylmethylsulfonyl fluoride. Concentrations of TNF-α and IL-6 were determined by use of commercially available immunoassay kits (Quantikine HS, R&D Systems).
Immunocytochemistry
Immunocytochemistry was performed on formalin-fixed paraffin-embedded sections to localize TNF-α and iNOS expression. Avidin-biotin-peroxidase complex method was used. Endogenous peroxidase was blocked with a solution of 0.03% vol/vol hydrogen peroxide in methanol for 20 minutes. After incubation with normal goat serum (1:30 for 30 minutes), sections were incubated overnight at 4°C with primary rabbit antibodies to TNF-α (Antigenex America Inc) diluted 1:100 and incubated overnight with primary rabbit antibodies to iNOS (Transduction Labs) diluted 1:400. Immunoreaction sites were visualized by use of appropriate biotinylated secondary antibodies and the avidin-biotin-peroxidase complex procedure (Vector Labs). Peroxidase activity was revealed with a solution of diaminobenzidine as chromogen with 0.2% vol/vol hydrogen peroxide in PBS to produce a brown reaction product and sections counterstained with Harris’ hematoxylin. Controls consisted of replacement of primary antibodies with nonimmune rabbit serum. Staining was graded 0 to 3 by two blinded independent observers.

Serum Immunoassay
Measurements of TNF-α, its receptors TNF-R1 and TNF-R2 and IL-6 were performed by use of commercially available immunoassay kits (Quantikine HS, R&D Systems).

Apoptotic Pathway
SDS-PAGE and Western Blotting
Myocardial tissue was homogenized in 1% SDS, 40 mmol/L phenylmethylsulfonyl fluoride, and total protein homogenates (30 μg) separated on 12%-T SDS-PAGE gels with a 3%-T stacking gel. Gels were equilibrated for 30 minutes in transfer buffer (20 mmol/L Tris base and 150 mmol/L glycine) and electrophoretically transferred to supported nitrocellulose (Hybond C Super) at 500 mA for 1 hour.

Detection of Cellular Proteins
Nitrocellulose membranes were blocked with 3% non-fat dried milk in PBS that contained 0.05% Tween 20 for 1 hour and then probed with primary antibodies against caspase-9, caspase-3, and substrates (PARP and DFF; Santa-Cruz Biotechnology). After they were washed, blots were incubated for another 1 hour in horseradish peroxidase–conjugated secondary antibodies (Dako). Protein bands were visualized by use of the Supersignal Ultra chemiluminescence substrate (Pierce).

Stripping Membranes
To stain tubulin after probing, blots were incubated in stripping solution (100 mmol/L 2-mercaptoethanol, 2% vol/vol SDS, and 62.5 mmol/L Tris-chloride; pH 6.7) for 30 minutes at 56°C. Membranes were washed and probed by use of tubulin with secondary horseradish peroxidase–conjugated antibodies. Reactive bands were detected as before.

Densitometry
Levels of expression of the procaspases, active caspases, and their substrates as assessed by immunoreactivity on ECL films was quantitated by laser densitometry and standardized to tubulin reactivity in each respective lane. Densitometric analysis was performed with Quant One software on a SunSparc station.

Cell Culture
Human U-937 myeloid leukemic cell line, treated separately with TNF-α at 4 mg/mL or staurosporine 1 μmol/L for 4 hours, and Jurkat T lymphoblastoid cell line, treated with 1 μmol/L of staurosporine for 6 hours, were used as positive controls.

Statistical Analysis
Variables are expressed as mean±SEM. Significance was assessed on grouped data with either Student’s t test or nonparametric Mann-Whitney U test. A P value <0.05 was considered significant.

Results

Cytokines

Myocardium
Tumor Necrosis Factor-α
TNF-α mRNA expression in myocardium of LVAD patients at time of implantation was 1.71-fold higher than in stable advanced heart failure patients (P<0.05, Figure 1A). TNF-α
protein content in myocardium of LVAD candidates (3.43 ± 0.19 pg/mg protein) was also higher than in advanced heart failure (2.95 ± 0.10 pg/mg protein; \( P < 0.05 \), Figure 1B). Myocardial TNF-α protein was slightly higher in those who died after LVAD implantation (3.57 ± 0.2 pg/mg protein) compared with those who survived >1 year (3.1 ± 0.2 pg/mg protein), although this did not reach statistical significance (\( P \geq NS \)).

**Interleukin-6**

Myocardial IL-6 mRNA expression was 2.57-fold higher in LVAD candidates than in stable advanced heart failure patients (9.78 ± 0.36 versus 1 ± 0.23; \( P < 0.001 \), Figure 3). IL-1β was 1.4-fold higher in those who died after LVAD implantation than in those that survived, but this did not reach statistical significance.

**Inducible Nitric Oxide Synthase**

iNOS mRNA expression in LVAD candidates (0.81 ± 0.19) was elevated to a level similar to that seen in advanced heart failure (1 ± 0.27), but iNOS mRNA was no higher in deteriorating patients compared with heart failure patients (Figure 4A).

**Serum**

Serum TNF-α was significantly higher in LVAD candidates (12.5 ± 1.9 pg/mL, \( n = 23 \)) compared with stable advanced heart failure patients (4.0 ± 0.4 pg/mL, \( n = 17 \); \( P < 0.0001 \), Figure 1E).

**Serum TNF-R1 and TNF-R2**

No significant difference was seen between serum TNF-R1 levels in LVAD candidates (2.9 ± 0.6 ng/mL, \( n = 23 \)) and heart failure patients (\( P < 0.05 \), Figure 2A). Myocardial IL-6 protein content was also significantly higher in LVAD candidates (27.83 ± 9.35 pg/mg protein) compared with advanced heart failure patients (4.26 ± 1.24 pg/mg protein; \( P < 0.001 \), Figure 2B). Myocardial IL-6 protein was significantly higher in those who died after LVAD implantation (38.94 ± 12.34 pg/mg protein) compared with those who survived >1 year (5.60 ± 1.64 pg/mg protein; \( P < 0.005 \)). These deaths occurred in patients who were on the device; no transplant-related deaths occurred.

**Interleukin-1β**

IL-1β mRNA expression was 9.78-fold higher in LVAD candidates than in stable advanced heart failure patients (9.78 ± 0.36 versus 1 ± 0.23; \( P < 0.001 \), Figure 3). IL-1β was 1.4-fold higher in those who died after LVAD implantation than in those that survived, but this did not reach statistical significance.

**TNF-α Immunocytochemistry**

TNF-α expression was immunolocalized predominantly to cardiac myocytes, although expression also was seen in endothelial cells and vascular smooth muscle cells of blood vessels. Myocyte expression of TNF-α in the LVAD candidates was significantly greater (mean score, 1.75 ± 0.20) than in advanced heart failure patients (mean score, 1.1 ± 0.16; \( P < 0.05 \), Figures 1C and 1D).

**Interleukin-6**

IL-6 mRNA expression in myocardium at time of LVAD implantation was 2.57-fold higher than in stable advanced heart failure patients (\( P < 0.005 \), Figure 2A). Myocardial IL-6 protein content was also significantly higher in LVAD candidates (27.83 ± 9.35 pg/mg protein) compared with advanced heart failure patients (4.26 ± 1.24 pg/mg protein; \( P < 0.001 \), Figure 2B). Myocardial IL-6 protein was significantly higher in those who died after LVAD implantation (38.94 ± 12.34 pg/mg protein) compared with those who survived >1 year (5.60 ± 1.64 pg/mg protein; \( P < 0.005 \)). These deaths occurred in patients who were on the device; no transplant-related deaths occurred.

**Inducible Nitric Oxide Synthase**

iNOS mRNA expression in LVAD candidates (0.81 ± 0.19) was elevated to a level similar to that seen in advanced heart failure (1 ± 0.27), but iNOS mRNA was no higher in deteriorating patients compared with heart failure patients (Figure 4A).

**iNOS Immunocytochemistry**

Significantly more iNOS expression was seen in blood vessels of the LVAD candidates (vascular smooth muscle staining, 1.75 ± 0.19 versus 0.75 ± 0.17; \( P < 0.005 \), Figures 4B and 4C), and slightly more iNOS staining was seen in myocytes of LVAD candidates (2.35 ± 0.15 versus 1.93 ± 0.13; which only just reached statistical significance; \( P < 0.05 \)).

**Serum**

Serum TNF-α was significantly higher in LVAD candidates (12.5 ± 1.9 pg/mL, \( n = 23 \)) compared with stable advanced heart failure patients (4.0 ± 0.4 pg/mL, \( n = 17 \); \( P < 0.0001 \), Figure 1E).

**Serum TNF-R1 and TNF-R2**

No significant difference was seen between serum TNF-R1 levels in LVAD candidates (2.9 ± 0.6 ng/mL, \( n = 23 \)) and...
stable advanced heart failure patients (2.8±0.3 ng/mL, n=17; P=NS). Serum TNF-R2 levels also were no higher in LVAD candidates (6.9±1.1 ng/mL, n=23) than in advanced heart failure patients (9.0±0.8 ng/mL, n=17; P=NS).

**Serum IL-6**
Serum IL-6 was higher in LVAD candidates (79.3±23.6 pg/mL, n=23) than in advanced heart failure patients (7.1±1.6 pg/mL, n=17; P<0.0001; Figure 2C).

**Apoptosis**

**Caspase-9**
Caspase-9 antibody recognizes the intact 46- to 48-kDa protein and the cleaved 37-kDa subunit. Levels of 46-kDa procaspase-9 were higher in LVAD candidates (8.02±0.91 oligodeoxynucleotide [OD] units) than in advanced heart failure patients (6.16±0.43 OD units; P<0.01, Figure 5A). Expression of 37-kDa cleaved caspase-9 was significantly higher in LVAD candidates (10.02±1.0 OD units) than in stable heart failure patients (7.34±0.40 OD units; P<0.05, Figure 5B).

**Caspase-3**
Caspase-3 antibody recognizes the intact 32-kDa protein and the 2 cleaved subunits of 11 and 20 kDa. Levels of procaspase-3 were not higher in LVAD patients (9.62±0.5 OD units) than in advanced heart failure patients (9.78±0.5 OD units; P=NS).

**Poly(ADP-Ribose) Polymerase**
PARP antibody recognizes both full-length 116-kDa protein and the cleaved 85-kDa fragment. Levels of intact PARP
were not significantly higher in LVAD candidates (0.37±0.08 OD units) than in stable advanced heart failure patients (0.27±0.05 OD units).

**DFF-45**

DFF-45 antibody (K-17) recognizes the intact 45-kDa inhibitor, the spliced 35-kDa product, and the cleaved 12-kDa product. Levels of intact DFF-45 were significantly higher in LVAD patients (10.86±0.91 OD units) than in advanced heart failure patients (8.91±0.38 OD units; \( P<0.05 \)). The spliced 35-kDa product also was significantly higher in LVAD candidates (4.58±0.75 OD units) than in advanced heart failure patients (2.84±0.23 OD units; \( P<0.05 \)).

**CPAN**

Levels of CPAN were not significantly higher in the LVAD candidates (2.04±0.5 OD units) than in advanced heart failure patients (2.57±0.23 OD units; \( P=NS \)).

**Discussion**

The present study has shown, for the first time, the specific pattern of increased expression of cytokines (Figure 6) together with alterations in the apoptotic pathway of the myocardium and serum of deteriorating patients who require implantation of an LVAD. We have shown elevated levels of myocardial TNF-\( \alpha \), IL-1\( \beta \), and IL-6 (Figure 6) in these patients compared with patients who have less severe heart failure, are undergoing transplantation, and have elevated levels of circulating TNF-\( \alpha \) and IL-6. Elevated myocardial expression of caspase-9 and DFF-45, but not caspase-3 and CPAN, in the final stage of the apoptotic pathway also was demonstrated.

An estimated 5 million people in the United States and 600 000 in the United Kingdom have heart failure. Mortality level for NYHA class III and IV heart failure is \( \approx 40\% \). The situation is currently worsened by the decreasing supply of donor organs. When heart failure patients deteriorate, insertion of an LVAD can be lifesaving. Early implantation of the device is beneficial to outcome; hence, finding clinical and molecular markers is important for the identification of patients at high risk of deterioration so that corrective action can be taken earlier.

We used real-time PCR to measure myocardial cytokine levels because it is a new, quantitative, highly reliable method. We chose to compare deteriorating patients to those in a stable heart failure group without using a donor control group as we have previously shown increased cytokine expression after brain death in donor hearts.\(^2\) The 13 patients in whom myocardial markers were measured had clinical parameters that showed them to be representative of the total group of patients. Limitation of tissue did not allow measurement of myocardial markers in all 23 patients.

In the present study, we found elevated levels of TNF-\( \alpha \) mRNA and protein in the myocardium of deteriorating patients who required LVAD insertion. TNF-\( \alpha \) is known to be elevated in heart failure,\(^1\)–\(^5\) and levels are known to correlate with NYHA functional class.\(^2\) Infusion of TNF-\( \alpha \) into rats at levels similar to levels present in end-stage heart failure results in depression of LV function, myocyte shortening, and LV dilatation.\(^6\) A recent study has shown increased myocardial TNF-\( \alpha \) with the progression of heart failure.\(^5\) Therefore, TNF-\( \alpha \) may be a useful marker of deterioration in these patients and also may be involved pathologically in the cause of the deterioration. In our present study, immunocytochemistry demonstrated TNF-\( \alpha \) expression in cardiac myocytes, and cardiac myocytes are known to be able to produce large amounts of TNF-\( \alpha \).\(^2\) TNF-\( \alpha \) acts on 2 cell-surface receptors, TNF-R1 and TNF-R2, which are thought to mediate and regulate most of the effects of TNF-\( \alpha \).\(^2\) These receptors are shed as soluble forms that are thought to act as buffers to neutralize the cytotoxic activity of TNF-\( \alpha \).\(^2\) The elevation of serum TNF-\( \alpha \) in the LVAD patients in our present study was not accompanied by a rise in TNF receptors. This suggests that the increased TNF-\( \alpha \) might not be neutralized by its receptors and, thus, may be able to act pathologically.

We have found elevated IL-6 mRNA and protein in the myocardium and elevated IL-6 in the serum of deteriorating patients who required LVAD insertion in the present study. IL-6 is known to be elevated in patients with heart failure, and raised levels correlate with decreased functional class, low ejection fraction, and poor prognosis.\(^1,3,7,8\) A recent study has shown increased myocardial IL-6 expression with progression of heart failure.\(^5\) Although IL-6 is known to increase with norepinephrine levels,\(^7\) none of the patients in the present study were on norepinephrine at the time of LVAD insertion. Thus, use of norepinephrine is unlikely to be the cause of elevated IL-6 in the present study. IL-6 can be negatively inotropic, and transgenic mice overexpressing the IL-6 gene develop ventricular hypertrophy and increased heart size.\(^2\) Of the deteriorating patients who underwent LVAD insertion in our present study, myocardial IL-6 protein expression was higher in those who died after the LVAD was implanted. Hence, IL-6 may be a good marker of deterioration and may be involved pathologically in deterioration (although elevation of IL-6 in the patients who died could imply irreversibility).

IL-1\( \beta \) negatively affects myocardial function\(^9,10\) and was 9-fold higher in the myocardium of deteriorating compared with stable patients (Figure 6). To our knowledge, this is the first time IL-1\( \beta \) has been shown to be elevated in the myocardium of patients with heart failure and to be quanti-
tatively increased in deteriorating patients. IL-1β may be a useful marker of deterioration and may be involved in its pathogenesis.

Both TNF-α and IL-1β induce iNOS expression, and both together act synergistically. Therefore, we investigated iNOS expression in these patients and found that although iNOS was elevated to a level similar to that seen in advanced heart failure patients, iNOS mRNA expression was not higher in deteriorating compared with stable patients. This suggests that iNOS is not the mechanism through which these 2 cytokines act in these patients. Immunocytochemistry showed elevated iNOS protein expression in myocytes, which suggests a possible increase at the translational level. However, this barely reached statistical significance. Given that immunocytochemistry alone is not a reliable quantitative method, we feel that these results should be interpreted with caution. Immunocytochemistry demonstrated strong iNOS staining in the vascular smooth muscle cells of intracardiac blood vessels of many LVAD candidates, which suggests that increased nitric oxide release occurs in blood vessels of deteriorating patients, which could contribute to their hypotensive state.

Both TNF-α and IL-1β can induce apoptosis, whereas IL-6 tends to inhibit it. We examined expression of caspases in the terminal stage of the apoptotic pathway to see whether they were elevated in deteriorating patients. Levels of procaspase-9 and activated, cleaved caspase-9 were elevated in these patients, but caspase-3 levels were not, which suggests a negative feedback mechanism at the level of caspase-3. Furthermore, levels of intact DFF-45 (inhibitor of DFF-45/CPAN) also were elevated, as was the cleaved 35-kDa product, but levels of the nucleases CPAN were not. Again, this occurrence suggests a negative feedback mechanism. IL-6 can increase BclXL and signal transducer and activator of transcription-3 (STAT-3). Thus, IL-6 tends to be antiapoptotic. Given that we have demonstrated it to be elevated in these patients, IL-6 may be part of the negative feedback mechanism. However, it is likely that several molecules are involved in both positive and negative feedback in the apoptotic pathway in these patients. Further investigation is required. For example, nuclear factor-κB expression is induced by TNF-α and IL-1β, is known to inhibit apoptosis, and is likely to be a potentially important mechanism. Lack of significant elevation in all of the terminal markers of apoptosis is difficult to explain and could be due to a negative feedback mechanism. Alternatively, our methods may have failed to detect a rise in these markers, or it may be that activation of the apoptotic pathway is not an important contributing mechanism to deterioration in these patients.

In conclusion, the present study has shown a specific pattern of cytokine elevation (Figure 6), alterations to caspases in the final stage of the apoptotic pathway of myocardium, and elevated levels of circulating cytokines in deteriorating patients who require LVAD insertion. Quantitative real-time RT-PCR demonstrated a 9-fold increase in IL-1β mRNA expression, elevated myocardial TNF-α and IL-6 mRNA and protein, and raised serum TNF-α and IL-6 in deteriorating patients. iNOS was elevated to levels similar to those of heart failure, but no further elevation occurred in the deteriorating patients, which suggests that iNOS is unlikely to be the pathway through which TNF-α and IL-1β act in these patients. Increased iNOS vascular smooth muscle expression was seen in blood vessels, which may contribute to the hypotension observed in these patients. Elevations were seen in procaspase-9, cleaved caspase-9, and intact and cleaved DFF-45 of the final part of the apoptotic pathway but not in caspase-3 and CPAN, which suggests that some negative feedback mechanisms are occurring in these patients that need further investigation.

These cytokines might be useful markers for the earlier detection of patients likely to need LVAD implantation. Serial serum cytokine measurements, supplemented if necessary by myocardial biopsy, could augment hemodynamic data and detect decompensation at an earlier stage.

Furthermore, blocking cytokine production may have the potential to prevent disease progression. Pentoxifylline is a xanthine derivative that suppresses or reduces production of TNF-α and is thought to act at the mRNA level. Our study suggests that administration of pentoxifylline to patients with end-stage heart failure potentially could prevent or reverse their deterioration. Etanercept is a p75 TNF-R fusion protein that binds to TNF-α, functionally inactivating it, which also may be effective for prevention of decompensation of such patients. Both of these drugs previously have been shown to produce clinical improvement in patients with less severe heart failure. Our present study suggests that IL-6 antagonists also potentially may prevent disease progression, as might IL-1 antagonists and specific caspase inhibitors, such as caspase-9 inhibitors. These approaches need to be investigated.

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