In vitro endothelial cell activation and inflammatory responses in end-stage heart failure

Ginette S. Hoare, Emma J. Birks, Christopher Bowles, Nandor Marczin, and Magdi H. Yacoub

Imperial College London, Heart Science Centre, Royal Brompton and Harefield National Health Service Trust, Harefield, Middlesex, United Kingdom

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Hoare, Ginette S., Emma J. Birks, Christopher Bowles, Nandor Marczin, and Magdi H. Yacoub. In vitro endothelial cell activation and inflammatory responses in end-stage heart failure. J Appl Physiol 101: 1466–1473, 2006. First published July 6, 2006; doi:10.1152/japplphysiol.01497.2005.—Background: vascular endothelial cell activation and dysfunction are observed in patients with severe heart failure and may contribute to systemic manifestations of this syndrome. It remains unknown whether inflammatory activation of these cells occurs in these patients because of increased circulating proinflammatory mediators. Aim: to determine whether the serum from patients with heart failure possesses a net proinflammatory bioactivity to active proinflammatory pathways in cultured endothelial cells. Methods: serum was obtained from stable patients with end-stage heart failure undergoing elective cardiac transplantation (Tx) and severely compensated patients with heart failure requiring emergency left ventricular assist device (LVAD) implantation. Net proinflammatory bioactivity of serum was investigated by monitoring ICAM-1 degradation and E-selectin expression in cultured human pulmonary artery endothelial cells (HPAEC) following incubation with serum samples. Serum cytokine concentrations were measured by ELISA and neutralizing antibodies were used to determine the role of specific factors in the observed bioactivity. Result: serum from both patient groups induced HPAEC ICAM-1 degradation. Low basal HPAEC E-selectin expression significantly increased following treatment with Tx but not LVAD serum. Serum tumor necrosis factor-α (TNF-α) and IL-10 concentrations were higher in patients with LVAD than those with Tx, and soluble TNF-α receptor expression was high in both groups. Neither TNF-α nor IL-10 blocking experiments altered either bioassay result. Conclusion: activation of a specific profile of pro- and anti-inflammatory mediators is associated with heart failure resulting in HPAEC nuclear factor (NF)-κB activation. However, E-selectin expression is further regulated by unidentified factors. TNF-α is upregulated but appears to play no part in NFκB activation in these patients. These findings could have important therapeutic implications.

inflammation; adhesion molecule; bioactivity; cytokine

HEART FAILURE is associated with the dysregulation of a number of neurohormonal systems including the adrenergic and renin-aldosterone-angiotensin system, the modulation of which form critical therapeutic strategies in the management of this syndrome. However, none of the existing therapeutic regimens employed prevent the ultimate progression of the disease and the deterioration of left ventricular (LV) function, suggesting that other systems are necessarily involved in this pathological process. Much of the current research aimed at elucidating the mechanisms of disease progression has focused on the involvement of proinflammatory cytokines and their potential as a therapeutic target in the treatment of heart failure. The cytokine theory of heart failure was born from the observation that expression of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and IL-6 are increased in both the myocardium and the circulation of patients with heart failure and correlate with deteriorating functional status and disease progression (4, 5, 13, 20, 23, 39). In addition, when expressed in sufficiently high concentrations, known biological effects of these molecules can explain many aspects of the heart failure phenotype (6, 7, 22, 29, 38, 44). As such, the syndrome of heart failure is generally considered to possess a clear inflammatory component that is of clinical significance not only locally in the myocardium but also systemically through the alteration of endothelial cell function, thus contributing to the circulatory adaptations that occur during heart failure.

The activation of the innate immune system is thought to play a major role in heart failure. Many tissues, including the heart and the vascular endothelium, may contribute to the production of cytokines. We hypothesize that, in addition to secretion of cytokines, endothelial cells are also an important target of such, and the inflammatory activation of these cells plays an important role in the progression of heart failure. Endothelial cells play a critical role in the control of vascular function. Under resting conditions, the vascular endothelium functions to inhibit platelet aggregation, leukocyte activation, and adhesion, prevent the proliferation of the underlying smooth muscle cells, prevent excessive deposition or degradation of matrix, and regulate vascular tone and permeability. Activation or injury to the endothelial cells by soluble factors in the circulation, interaction with activated blood cells, altered flow conditions, and shear stress will change their function and alter the balance between the many countervailing forces they control to maintain homeostasis within the blood vessel.

Several studies have demonstrated that the serum of patients with heart failure can alter the function of endothelial cells by downregulating the expression of endothelial nitric oxide synthase and inducing endothelial cell apoptosis in culture (2, 33). Furthermore, using a novel endothelial cell biopsy technique, Colombo et al. (9, 10) recently demonstrated peripheral vein endothelial cell activation (inducible nitric oxide synthase and cyclooxygenase 2 expression) and impaired endothelial cell function in patients with decompensated heart failure that subsides with a return to a clinically compensated state. Indeed, impaired vascular endothelial function is associated with increased mortality risk in heart failure (21).

However, given the importance put on the role of proinflammatory factors in the progression of heart failure, little is known about the proinflammatory potential in the circulation of these patients with regard to the ability to activate proinfla-
HEART FAILURE AND ENDOTHELIAL CELL ACTIVATION

flamatory pathways in endothelial cells. The aim of this study was to determine whether serum from patients with heart failure possess the bioactivity to activate inflammatory pathways in endothelial cells in culture using a bioassay based on the activation of the nuclear factor (NF)-kB pathway and induction of endogenous inflammatory gene expression. Although heart failure is a progressive disease, a subset of patients at a certain point in time develop a very rapid progressive deterioration that requires inotropic support and emergency implantation of a left ventricular assist device (LVAD). The cause of such is unknown. We hypothesize that changes in the balance between pro- and anti-inflammatory cytokines could play an important role. To clarify this, we have compared these patients’ profiles with patients with end-stage heart failure who are clinically stable.

MATERIALS AND METHODS

Study Design

Two groups of patients with heart failure were recruited to this study on the basis of the severity of disease and the operative treatment required for each (elective cardiac transplantation vs. emergency LVAD implantation). Endothelial cell activation in response to treatment with the serum samples from these patients was investigated in an in vitro bioassay system by monitoring the ability of each sample to initiate the activation of NF-kB and to induce the expression of E-selectin in pulmonary artery endothelial cells. These cells were chosen because of their strategic location. It is likely that other types of endothelial cells will react in a similar fashion, although endothelial cell function is known to be heterogeneous. In an attempt to identify the serum factors responsible for the observed bioactivity, serum levels of individual pro- and anti-inflammatory cytokines were measured and antibody blocking studies were performed.

The protocol for this study was approved by the Royal Brompton and Harefield Research Ethics committee and informed consent was obtained from each patient.

LVAD Patients

The study included 14 consecutive patients undergoing emergency LVAD implantation because of deteriorating clinical status with evidence of secondary organ dysfunction in the context of low cardiac output and increased filling pressure despite appropriate medical treatment. All 14 patients were male. Their mean age was 38.0 ± 4.3 yr. They were diagnosed as follows: dilated cardiomyopathy (11), ischemic heart disease (1), ischemic heart disease with postinfarct ventricular septal defect rupture (2). All patients were in New York Heart Association (NYHA) Class IV and had deteriorated over a period of 2.3 ± 0.4 days. All patients were on inotropes and eight were supported by intra-arterial balloon pump (IABP; Table 1). Patients with histological evidence of myocarditis were excluded from the study. Blood was collected immediately before LVAD insertion, centrifuged at 2,500 rpm for 10 min, and the resulting serum supernatants were stored at −40°C.

Heart Failure Patients

The study included 15 consecutive patients with stable advanced heart failure undergoing elective cardiac transplantation and not meeting our criteria for LVAD implantation. Thirteen patients were male and two were female. Their mean age was 45.3 ± 3.7 yr. Their diagnosis was as follows: dilated cardiomyopathy (8), ischemic heart disease (6), postpartum cardiomyopathy (1). Twelve patients were in NYHA Class III and three in NYHA Class IV. Three patients were on inotropes and one was supported by IABP. Of the remaining patients, 11 were receiving angiotensin I-converting enzyme inhibitor treatment and one the angiotensin receptor blocker losartan (Table 1). Patients with histological evidence of myocarditis were excluded from the study. Blood was collected at the time of cardiac transplantation, centrifuged at 2,500 rpm for 10 min, and the resulting serum supernatants were stored at −40°C.

Cell Culture

Human pulmonary artery endothelial cells (PAEC) were isolated from the pulmonary artery修剪ings from heart and heart/lung donors. Cells were grown on 1% gelatin in M199 medium supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml), l-glutamine (2 mM), 25% heat-inactivated fetal bovine serum, 25 mM HEPES, and endothelial cell growth factor. For experiments, cells were seeded into 24-well plates and allowed to grow to 80% confluence. Cells were incubated with M199 containing no additives for 24 h before the start of each experiment to achieve a true resting state. Under these conditions, cellular IkBα protein levels were abundant, there was no evidence of nuclear NF-kB p65 (data not shown), and E-selectin expression remained low.

Bioassay

Influence of patients’ serum on PAEC IkBα levels. IkBα levels in cultured PAEC were monitored in response to patient serum as a measure of the ability of inflammatory factors to activate NF-kB. PAEC were incubated with 10 µM cycloheximide for 30 min before incubation with vehicle, recombinant human TNF-α (rTNF-α; 0.001–10 ng/ml), or serum (diluted 1:1 in medium). For TNF-α neutralizing experiments, the TNF-α neutralizing antibody (0.5 µg/ml, R&D Systems) was added to the serum or cytokines and incubated at 37°C for 30 min before addition to cells. Following treatment, cells were lysed in 1% SDS containing protease inhibitors. Proteins were resolved on 10% SDS-polyacrylamide gel by electrophoresis and transferred to nitrocellulose membrane. Proteins were detected using polyclonal anti-IkBα (0.2 µg/ml, Santa Cruz (sc 371)) or monoclonal anti-β-tubulin (clone TUB2.1), 1.8 µg/ml, Sigma T4026) and relevant horseradish peroxidase-conjugated secondary antibody (Dakocytomation; P0448, P0260). Immunoreactive bands were visualized using enhanced chemiluminescence (ECL) detection system (Amersham Biosciences). Films were scanned using Bio-Rad GS-710 densitometer and analyzed using BioRad Quantity One software.

Influence of patients’ serum on PAEC E-selectin expression. E-selectin expression on PAEC was monitored in response to patient serum as a measure of induction of a NF-kB regulated endogenous gene. PAEC were incubated for 5 h with vehicle, rTNF-α (0.001–10 ng/ml), or patient serum (diluted 1:1 with medium). For anti-TNF-α and anti-IL-10 blocking studies, neutralizing antibody (anti-TNF-α

Table 1. Characteristics of study patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>LVAD</th>
<th>Tx</th>
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<tr>
<td>Age, yr (mean ± SE)</td>
<td>38.0 ± 4.3</td>
<td>45.3 ± 3.7</td>
</tr>
<tr>
<td>Male/Female (no. of patients)</td>
<td>14/0</td>
<td>13/2</td>
</tr>
<tr>
<td>NYHA Class III/IV (no. of patients)</td>
<td>0/14</td>
<td>12/3</td>
</tr>
<tr>
<td>Diagnosis (no. of patients)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dilated cardiomyopathy</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td>IHD</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>IHD with postinfarct ventricular septal defect rupture</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Postpartum cardiomyopathy</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Number of patients on Inotropes</td>
<td>14</td>
<td>3</td>
</tr>
<tr>
<td>Number of patients with IABP</td>
<td>8</td>
<td>1</td>
</tr>
</tbody>
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LVAD, left ventricular assistive device; Tx, cardiac transplant; IHD, ischemic heart disease; IABP, intra-arterial balloon pump.
0.5 μg/ml R&D Systems or anti-IL-10 1 μg/ml R&D Systems, MAB217) was added to the serum or cytokines and incubated at 37°C for 30 min before addition to cells. Following treatment, cells were trypsinized and E-selectin expression determined by flow cytometry using anti-E-selectin antibody (2 μg/ml, clone BBIG-E5, R&D Systems, BBA21) and FITC-conjugated secondary antibody (Dakocytomation, F0313). Log fluorescence intensity was measured and gates set at 2% positive cells on isotype control.

Analysis of Serum Cytokine Concentrations

Serum cytokine levels were measured using commercially available immunoassays (R&D Systems).

Statistical Analysis

Results involving patient samples did not pass normality and/or equal variance testing and thus the data cannot be assumed to be normally distributed. These results are therefore expressed as median values with ranges. Differences between groups are assessed using Mann-Whitney rank sum test or Kruskal-Wallis one-way ANOVA on ranks followed by Dunn’s post hoc test. Box plots represent median and 10th, 25th, 75th, and 90th percentiles. For bioassay data, at least three independent assays, using pulmonary artery endothelial cell isolates from different sources, were performed for each patient sample and the mean response for each sample was used for grouped comparisons.

RESULTS

Effect of Patient Serum on Endothelial Cells IκBα Levels

Control AB serum had no significant effect on PAEC IκBα levels (94.1 ± 21.0% of control levels), whereas 30-min treatment of these cells with increasing concentrations of TNF-α (1 pg/ml–10 ng/ml) induced a concentration-dependant reduction in the level of IκBα protein in these cells that was significant at 10 pg/ml (51.0 ± 6.0% of control levels, P < 0.05, n = 7, Fig. 1A).

Compared with the AB serum control, incubation of PAEC with patient serum for 60 min induced a significant degradation of IκBα the remaining levels of which were similar to those following treatment with TNF-α (10 pg/ml). IκBα levels in PAEC were reduced to 42.2% (27.8–74.4) of that in control cells following treatment with serum from patients undergoing heart failure.

Fig. 1. Effect of recombinant human tumor necrosis factor (TNF)-α and serum of patients with heart failure of IκBα levels in cultured human pulmonary artery endothelial cells. A: summary graph of the densitometric analysis of Western blots demonstrating the degradation of IκBα protein from pulmonary artery endothelial cells (PAEC) following 30-min treatment with increasing concentrations of recombinant TNF-α. B: representative Western blots showing the bioactivity of 6 cardiac transplant (Tx) and 6 left ventricular assistive device (LVAD) serum samples to induce PAEC IκBα degradation. C: summary graph of the densitometric analysis of IκBα Western blots demonstrating the grouped bioactivity of Tx and LVAD patients’ serum to induce IκBα degradation in PAEC. *P < 0.05 from control treated cells.
cardiac transplantation \((n = 10, P < 0.05)\) and to 35.7\% (31.6–72.8) following treatment with serum from LVAD patients \((n = 10, P < 0.05)\). There was no significant difference between the extent of I\(\kappa\)B\(\alpha\) degradation induced by serum from either Tx or LVAD patients \((P = 0.623, \text{Fig. 1, B and C})\).

**Effect of Patient Serum on Endothelial Cell E-Selectin Expression**

Treatment of the PAEC with control AB serum had no significant effect on E-selectin expression [3.8 ± 0.4% positive cells \((n = 8)\) vs. 3.3 ± 0.3% in control resting cells \((n = 19, P = 0.391)\)].

Treatment of PAEC with Tx patient serum caused a small but significant increase in E-selectin expression [5.6\% (3.2–9.4) positive cells, \(P < 0.05, n = 15\)] above control levels [2.9\% (2.1–7.7) positive cells]. There was, however, no significant increase in E-selectin expression above control levels following incubation of the cells with LVAD patient serum [3.1\% (2.3–9.8) positive cells, \(P > 0.05, n = 14, \text{Fig. 2}\)].

Treatment of these PAEC with 1 and 10 pg/ml TNF-\(\alpha\) induced a significant increase in E-selectin expression above control levels.

**Fig. 3. Serum cytokine concentrations in deteriorating patients with heart failure undergoing emergency LVAD implantation compared with patients with stable advanced heart failure undergoing elective Tx.**

- **A**: TNF-\(\alpha\); **B**: molar ratio of type 1 soluble TNF-\(\alpha\) receptor (sTNFR1)/TNF-\(\alpha\); **C**: molar ratio of sTNFR2/TNF-\(\alpha\); **D**: IL-1\(\beta\); **E**: IL-10; **F**: IL-6 Box plots depict median values with 10th, 25th, 75th, and 90th percentiles. [Mean plasma cytokine concentrations in healthy controls (data from R&D systems ELISA handbooks): TNF-\(\alpha\) 1.98 pg/ml, IL-1\(\beta\) <3.9 pg/ml, sTNFR1 914 pg/ml, sTNFR2 1,500 pg/ml, IL-10 <7.8 pg/ml, IL-6 1.49 pg/ml].

**Fig. 4. Effect of anti-TNF-\(\alpha\) neutralizing antibody on I\(\kappa\)B\(\alpha\) depletion in PAEC.** Western blot demonstrating the inhibitory effect of anti-TNF-\(\alpha\) antibodies on TNF-\(\alpha\) but not IL-1\(\beta\)-induced I\(\kappa\)B\(\alpha\) depletion.

**Fig. 2. Effect of serum from Tx and LVAD patients on E-selectin expression on PAEC.** Summary graph of grouped data demonstrating the bioactivity of serum of patients with heart failure to induce E-selectin expression on PAEC in culture compared with the effects of low concentrations of TNF-\(\alpha\) and control AB serum. *\(P < 0.05\) from control untreated cells.
levels [7.1% (2.0–45.8) positive cells, n = 7 and 8.0% (2.1–66.3) positive cells, n = 12, Fig. 2].

Cytokines

Similar to other studies, TNF-α levels were found to be increased in the circulation of the severely deteriorating LVAD patients [8.5 pg/ml (4.1–32.9), n = 14] compared with the patients with less severe heart failure undergoing cardiac transplantation [3.6 pg/ml (1.8–7.8), n = 15, P < 0.05; Fig. 3A].

Serum concentrations of type 1 soluble TNF-α receptor (sTNFR1) and type 2 soluble TNF-α receptor (sTNFR2) did not significantly differ between the LVAD and the Tx patients [2,833.0 pg/ml (518.0–6794.0) vs. 3,059.2 pg/ml (227.7–9,623.2), P = 0.556 and 9.087.0 pg/ml (2,203.0–17,409.0) vs. 5,428.8 pg/ml (1,577.5–25,190.4), P = 0.305, respectively]. However, the molar ratio of serum sTNFR1 to TNF-α was significantly lower in the LVAD patients [318.7 (21.5–527.6)] compared with the Tx patients [435.4 (90.7–1,047.1), P = 0.009, Fig. 3B] as was the molar ratio of sTNFR2 to TNF-α [306.6 (100.9–789.2) vs. 1,055.8 (289.3–2,305.9), P < 0.001; Fig. 3C].

Circulating IL-1β levels were low in all patients and not significantly different between the two groups (P = 0.964; Fig. 3D).

Serum IL-10 levels were significantly higher in LVAD patients [12.1 pg/ml (0.0–85.9), n = 14] compared with Tx patients [1.7 pg/ml (0.0–30.4), n = 12, P = 0.016, Fig. 3E]. Serum IL-6 was significantly higher in LVAD [91.0 pg/ml (6.3–129.8), n = 14] compared with Tx patients [3.6 pg/ml (0.7–15.6), n = 12, P < 0.001, Fig. 3F].

DISCUSSION

This study has demonstrated that the serum of patients with end-stage heart failure does possess the bioactivity to initiate the activation of the inflammatory transcription factor NF-κB in vascular endothelial cells in culture, demonstrating a proinflammatory state. It has also confirmed the association of TNF-α and IL-10 with the activation of endothelial cells in vivo, as measured by the increased expression of E-selectin.

Investigation Into the Involvement of TNF-α in the Observed Bioactivity

To investigate the potential importance of TNF-α in the observed proinflammatory bioactivity of the serum, the bioassays were performed in the presence of anti-TNF-α neutralizing antibody. Under conditions that significantly inhibited TNF-α but not IL-1β-induced IkBα depletion in PAEC (Fig. 4), the presence of anti-TNF-α neutralizing antibodies failed to alter the effects of serum from either Tx (P = 0.571) or LVAD (P = 0.850) patients on PAEC IkBα levels (Table 2).

Table 2. Effect of anti-TNF-α antibodies on IkBα levels in cells treated with Tx or LVAD patient serum

<table>
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<tr>
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<th>Control</th>
<th>Anti-TNF-α</th>
<th>P Value</th>
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<tr>
<td>Tx</td>
<td>61.5 (23.7–151.0)</td>
<td>56.4 (27.7–136.8)</td>
<td>0.574</td>
</tr>
<tr>
<td>LVAD</td>
<td>51.2 (20.9–116.7)</td>
<td>52.6 (27.9–122.9)</td>
<td>0.694</td>
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Values are medians with range in parentheses. TNF-α, tumor necrosis factor-α; LVAD, left ventricular assistive device. IkBα levels are expressed as a % of control levels.

In agreement with this result, there was no significant correlation between TNF-α levels or sTNFR/TNF-α molar ratios and bioactivity to induce IkBα depletion or increased E-selectin expression in either group of patients (P > 0.05).

Involvement of IL-10 in the Observed Bioassay

There was a significant negative correlation between bioactivity to induce E-selectin expression and serum IL-10 concentrations (r² = 0.432, P < 0.001).

To explore the influence of IL-10 on E-selectin expression, the bioassay experiments were carried out in the presence of anti-IL-10 neutralizing antibodies. Under conditions that specifically attenuated IL-10 (50 ng/ml)-induced signaling events (JAK1 and STAT1 phosphorylation), in a responsive cell line (U937, Fig. 6A and B), neutralizing IL-10 failed to alter the bioactivity of either Tx or LVAD serum with respect to E-selectin induction [3.8% (2.7–5.5), P = 0.970, n = 10 and 2.2 (0.74–6.1), P = 0.909, n = 14 positive cells, respectively; Fig. 5B].

Fig. 5. Effect of anti-TNF-α neutralizing antibody on E-selectin expression in PAEC. A: graph demonstrating the inhibitory effect of anti-TNF-α antibodies on TNF-α but not IL-1β-induced increase in E-selectin. B: summary graph showing the lack of effect of TNF-α neutralizing antibody on E-selectin expression in PAEC following treatment with Tx and LVAD patient serum.
LVAD patients to induce E-selectin expression. IL-10 activity with anti-IL-10 antibodies on the bioactivity serum from Tx and LVAD patients. How-ever, serum-induced IkBα degradation induced by treatment of the endothelial cells with serum of both groups of patients were similar despite the concentration of TNF-α being higher in the decompensated LVAD patients. Second, any effect of TNF-α must take place in the context of simultaneously present factors that may either enhance or inhibit its biological effect. In this regard, circulating levels of sTNFR in both groups of patients were increased compared with normal levels (1), and the high molar excess of sTNFR to TNF-α in all these patients suggests that the biological activity of TNF-α may be effectively neutralized (15, 18, 40). This theory was strengthened by TNF-α blocking studies that failed to alter the bioactivity of the serum to induce IkBα degradation. This negative finding regarding the influence of serum TNF-α in patients with heart failure on NF-κB activation is supported by a recent study by Frantz et al. (17) who demonstrated that increased NF-κB activation in the myocytes, endothelial cells, and infiltrating leucocytes in the failing myocardium also did not correlate with TNF-α levels. Taken together, these data suggest that factors other than TNF-α are important with regard to the activation of NF-κB in the setting of heart failure and further studies are required to identify them.

Given the level of IkBα depletion following treatment of PAEC with serum from Tx and LVAD patients and the essential requirement for NF-κB activation in conferring the efficient induction of the E-selectin gene, it was surprising that the expression of this adhesion molecule, in response to treatment with patients’ serum, was not increased to a greater extent. Serum from the LVAD patients failed to induce any significant increase in E-selectin expression, whereas from Tx patients induced a small but significant increase above control levels. These data suggest the presence of a significant counteracting anti-inflammatory activity in the circulation of the severely deteriorated LVAD patients that is capable of inhibiting the progression of endothelial cell NF-κB activation through to efficient transcription and translation of the E-selectin gene.

The successful induction of many NF-κB inducible genes requires the cooperative action of other inducible signals, transcription factors, and cofactors that allow the successful reorganization of the chromatin architecture, posttranslational modifications of NF-κB subunits, and the assembly of a transcriptionally competent enhansosome (14, 26, 34, 35, 41). Therefore there are numerous potential places within the sequence of events from IkBα depletion to E-selectin protein expression where an interruption in this pathway could occur.

In this study, we identified IL-10 as a potential candidate responsible for this anti-inflammatory activity. IL-10 expression has been previously observed in patients with heart failure (43) and serum levels measured in this current study were increased in the patients with LVAD compared with the patients with Tx and correlated with reduced bioactivity of the serum to induce E-selectin expression. IL-10 has been shown to inhibit NF-κB DNA binding activity in a number of cell

**Fig. 6.** Role of IL-10 in the observed bioactivity to induce E-selectin expression. A and B: Western blot demonstrating the specific inhibitory activity of anti-IL-10 neutralizing antibody on IL-10 but not IFN-γ signaling events (A, JAK1 phosphorylation; B, STAT1 phosphorylation). C: effect of neutralizing IL-10 activity with anti-IL-10 antibodies on the bioactivity serum from Tx and LVAD patients to induce E-selectin expression.

flammatory potential in the circulation of these patients. However, serum-induced IkBα degradation and thus the liberation of the NF-κB dimer did not always translate into efficient transactivation of the E-selectin gene, a process that relies heavily on efficient activation of NF-κB (11). These data suggest the presence of an anti-inflammatory activity capable of preventing the induction of the E-selectin gene by either interfering with the NF-κB pathway or an essential cooperating pathway.

The identity of the NF-κB activating bioactivity in the circulation of these patients with heart failure is at present unclear. The proinflammatory cytokine TNF-α is a tempting candidate, because it is a strong inducer of NF-κB activity in cultured endothelial cells (Fig. 3) and increased levels have been demonstrated in the circulation of patients with heart failure in this and other studies (4, 5, 13, 20, 23, 39). However, further investigations into the contribution of TNF-α to this inflammatory bioactivity of serum of patients with heart failure revealed that it is unlikely to be involved. First, the level of IkBα degradation induced by treatment of the endothelial cells with serum of both groups of patients were similar despite the concentration of TNF-α being higher in the decompensated LVAD patients. Second, any effect of TNF-α must take place in the context of simultaneously present factors that may either enhance or inhibit its biological effect. In this regard, circulating levels of sTNFR in both groups of patients were increased compared with normal levels (1), and the high molar excess of sTNFR to TNF-α in all these patients suggests that the biological activity of TNF-α may be effectively neutralized (15, 18, 40). This theory was strengthened by TNF-α blocking studies that failed to alter the bioactivity of the serum to induce IkBα degradation. This negative finding regarding the influence of serum TNF-α in patients with heart failure on NF-κB activation is supported by a recent study by Frantz et al. (17) who demonstrated that increased NF-κB activation in the myocytes, endothelial cells, and infiltrating leucocytes in the failing myocardium also did not correlate with TNF-α levels. Taken together, these data suggest that factors other than TNF-α are important with regard to the activation of NF-κB in the setting of heart failure and further studies are required to identify them.

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dissociation between the bioactivity of serum to induce limited amounts of CREB-binding protein (CBP) (31). Taken together, these studies demonstrated that increasing intracellular cAMP by inhibiting its breakdown. Early studies demonstrated that increasing intracellular cAMP with pharmacological agents could inhibit cytokine-induced expression of a number of NF-κB responsive genes in HUVEC, including E-selectin (16, 19, 28, 32). Importantly, increased intracellular cAMP had no effect on NF-κB nuclear translocation but inhibited its transcriptional activity through cAMP response element binding protein (CREB) successfully outcompeting NF-κB for binding to limited amounts of CREB-binding protein (CBP) (31). Taken together, this evidence provides a possible explanation for the dissociation between the bioactivity of serum to induce IkBα degradation and that to induce E-selectin expression in the patients with LVAD, all of whom were receiving such inotropic therapy. However, further analysis in this regard is severely hindered by the use of multiple combinations of such drugs on a relatively small number of patients and a dedicated follow-up study is needed to specifically address this issue.

In conclusion, this study has demonstrated that although proinflammatory cytokine expression is increased in the circulation of deteriorating patients with advanced heart failure, the consequences of such cannot be predicted when individual factors are viewed in isolation because there is also an increase in counteracting anti-inflammatory factors able to prevent some specific inflammatory events in peripheral endothelial cells. To the extent that this in vitro data may be extrapolated to the in vivo situation increased TNF-α and NF-κB activation in the setting of heart failure may not be adversely contributing to the pathology of heart failure and the rapid deterioration of the LVAD patients by proinflammatory mechanisms. Given that trials of anti-TNF-α and anti-inflammatory strategies in the treatment of heart failure have been largely unsuccessful (3, 8, 12, 25), future therapeutic strategies for the treatment of this syndrome may be of greater benefit when aimed at specific cellular events rather than the actions of single molecules or a generalized anti-inflammatory approach.

GRANTS

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