**ORIGINAL CLINICAL SCIENCE**

**Tumor necrosis factor-α levels and non-surgical bleeding in continuous-flow left ventricular assist devices**

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**KEYWORDS:**
- angiopoietin-2;
- angiogenesis;
- inflammation;
- thrombosis;
- non-surgical bleeding;
- mechanical circulatory support;
- tissue factor

**BACKGROUND:** Non-surgical bleeding (NSB) due to angiodysplasia is common in left ventricular assist device (LVAD) patients. Thrombin-induced angiopoietin-2 (Ang-2) expression in LVAD patients leads to altered angiogenesis and is associated with lower angiopoietin-1 (Ang-1) and increased NSB. However, the mechanism for decreased Ang-1, made by pericytes, is unknown and the origin of thrombin in LVAD patients is unclear. We hypothesized that high tumor necrosis factor-α (TNF-α) levels in LVAD patients induce pericyte apoptosis, tissue factor (TF) expression and vascular instability.

**METHODS:** We incubated cultured pericytes with serum from patients with heart failure (HF), LVAD or orthotopic heart transplantation (OHT), with or without TNF-α blockade. We performed several measurements: Ang-1 expression was assessed by reverse transcript-polymerase chain reaction (RT-PCR) and pericyte death fluorescently; TF expression was assessed by RT-PCR in cultured endothelial cells incubated with patient plasma with or without TNF-α blockade; and TF expression was assessed in endothelial biopsy samples from these patients by immunofluorescence. We incubated cultured endothelial cells on Matrigel with patient serum with or without TNF-α blockade and determined tube formation by microscopy.

**RESULTS:** Serum from LVAD patients had higher levels of TNF-α, suppressed Ang-1 expression in pericytes, and induced pericyte death, and there was accelerated endothelial tube formation compared with serum from patients without LVADs. TF was higher in both plasma and endothelial cells from LVAD patients, and plasma from LVAD patients induced more endothelial TF expression. All of these effects were reversed or reduced with TNF-α blockade. High levels of TNF-α were associated with increased risk of NSB.

**CONCLUSIONS:** Elevated TNF-α in LVAD patients is a central regulator of altered angiogenesis, pericyte apoptosis and expression of TF and Ang-1.

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Non-surgical bleeding (NSB) due to angiodysplasia is common among patients with continuous-flow left ventricular assist devices (LVADs). However, the underlying mechanism remains unknown. Angiodysplasia is associated with endothelial proliferation and decreased vessel coverage by pericytes, non-endothelial vascular cells that support the endothelium. Pericytes produce angiopoietin-1 (Ang-1), an agonist of Tie-2, which promotes vessel stability. Ang-1 is antagonized by angiopoietin-2 (Ang-2), which is synthesized by endothelial cells in response to thrombin. Ang-2 induces endothelial destabilization and altered vessel growth. We have
shown that Ang-1 is decreased in LVAD patients, although the underlying mechanism is unknown. We have also shown that thrombin-dependent overexpression of Ang-2 in LVAD patients induces altered angiogenesis and is associated with NSB. Because Ang-1 and Ang-2 are regulators of vascular growth, determining the mechanisms underlying the loss of Ang-1 and overexpression of Ang-2 in LVAD patients is critical to understanding LVAD-related angiodysplasia and NSB.

Tumor necrosis factor-α (TNF-α) induces pericyte apoptosis, promotes altered angiogenesis in synergy with Ang-2, and regulates Ang-2 expression. TNF-α also induces endothelial expression of tissue factor (TF, Factor III), which leads to thrombin production and is augmented in synergy with thrombin itself. Plasma TNF-α and TF are elevated after LVAD implantation and experts have theorized that TF may drive angiodysplasia in LVAD patients through thrombin and Ang-2. However, the interplay of TNF-α with angiogenic and coagulation pathways in LVAD patients is not known. We hypothesized that high levels of TNF-α in LVAD patients induce pericyte apoptosis, decrease Ang-1 expression, induce endothelial TF and Ang-2 expression, and promote altered angiogenesis.

Methods

Study subjects

We performed a cross-sectional study of patients with an LVAD (HeartMate II or HVAD), patients with heart failure (HF) with reduced ejection fraction without an LVAD, and patients with a history of orthotopic heart transplantation (OHT). Inclusions, exclusions and timing of recruitment were reported previously and are addressed in the Supplementary Material (available online at www.jhltonline.org/). The protocol was approved by the institutional review board of the University of Chicago and all participants provided written informed consent.

Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Basel, Switzerland), and human brain pericytes were purchased from ScienCell (Carlsbad, CA). Cultures were grown to 70% confluence under standard conditions (37°C, 5% CO₂).

Measurement of effect of TNF-α and associated molecules on vascular cells

These experiments are described in the Supplementary Material (online).

Measurement of circulating biomarkers

TNF-α and TF were measured by enzyme-linked immunoassay (ELISA; TNF-α: Life Technologies; TF: R&D Systems) in platelet-poor plasma.

Assessment of effect of TNF-α on Ang-1 expression in pericytes

Serum samples from patients with LVADs were diluted 1:1 with Dulbecco’s modified Eagle medium (DMEM), mixed with TNF-α-blocking antibody (100 ng/ml; Cell Signaling) or vehicle, and incubated at 37°C for 2 hours with intermittent mixing to allow neutralization of TNF-α. For this assay, we did not include serum from patients with OHT, because the OHT patients in our cohort were treated with tacrolimus, which may confound Ang-1 expression. After 2 hours, the mixture was pipetted onto dishes of cultured pericytes that were incubated for an additional 4 hours. Pericyte RNA was isolated using a PureLink RNA Mini Kit (Life Technologies) and gene expression was measured by reverse transcript-polymerase chain reaction (RT-PCR).

Assessment of effect of TNF-α on pericyte cell death

Serum samples from each patient were diluted 1:1 with DMEM and mixed with TNF-α-blocking antibody or vehicle as noted previously. After 2 hours, the mixture was pipetted onto cultured pericytes growing in 96-well plates, which were incubated for an additional 12 hours. Cultures were then stained with a calcine/ethidium viability kit (Fisher) and viability was determined on a plate reader. To control for variation in seeding density, pericyte death was measured as the ratio of dead/live cells in each well.

Measurement of TF expression in endothelial cells from patients

We obtained vena caval endothelial cells from guide-wires used during right heart catheterization and measured TF expression by quantitative immunofluorescence, as described elsewhere. Expression is presented in arbitrary units (AU).

Assessment of effect of TNF-α on endothelial expression of TF and Ang-2

Plasma samples from each patient were mixed with TNF-α-blocking antibody or vehicle as noted previously. After 2 hours, the mixture was pipetted onto dishes of cultured HUVECs, which were incubated for an additional 4 hours. HUVEC RNA was isolated as noted previously and gene expression was measured by RT-PCR.

Assessment of angiogenic potential of patients’ serum

Serum samples from each patient were diluted 1:1 with culture medium as described elsewhere and mixed with TNF-α-blocking antibody or vehicle, as noted previously. Then 24-well plates (Falcon) were coated with Matrigel (Corning) and solidified. Next, 200,000 HUVECs were resuspended in the serum/medium mixture from each patient. This mixture was pipetted into the Matrigel-coated wells and incubated overnight. Microtube formation was assessed by microscopy.

Assessment of Rho kinase activity

Previous studies have shown other inflammatory factors besides TNF-α may be elevated after LVAD implantation. We therefore
hypothesized that Rho kinase (ROCK) may also be elevated. We measured ROCK activity in these patients using a method we described in a previous study. Briefly, leukocytes were isolated from blood, protein was extracted, and ROCK activity was measured by Western blot.

Measurement of non-surgical bleeding outcomes

Patients were monitored for NSB events for 1 year after sample collection, or until they received an OHT or died (if within 1 year). NSB was defined as gastrointestinal bleeding, intracranial hemorrhage or epistaxis, as previously described.

Statistical analyses

Data were analyzed using SPSS version 23.0 (IBM SPSS). Continuous variables were compared between cohorts using the Mann–Whitney U-test or Kruskal–Wallis test, followed by pairwise post-hoc comparisons using the Mann–Whitney U-test with Bonferroni’s adjustment when positive. Treatment conditions were compared within cohorts using Wilcoxon’s signed-rank test. Categorical variables were compared using Fisher’s exact test. Kaplan–Meier distributions were compared using the log-rank test. Data are presented as mean ± standard deviation unless otherwise indicated. A 2-sided p < 0.05 was considered statistically significant.

Results

We enrolled 32 patients with HF, 44 patients with LVADs and 25 patients with OHT. Clinical characteristics are shown in Table 1 and Tables S1 to S4 (see Supplementary Material).

Elevated circulating TNF-α in plasma from LVAD patients

We measured TNF-α in platelet-poor plasma. TNF-α was significantly higher in patients with LVADs compared with HF or OHT patients (5.97 ± 4.42, 3.61 ± 3.31 and 3.05 ± 3.64 pg/ml, respectively; omnibus, p < 0.01; HF vs LVAD, p < 0.05; LVAD vs OHT, p < 0.05; HF vs OHT, p = not statistically significant [NS]).

Elevated TNF-α in serum from LVAD patients suppresses Ang-1 gene expression and induces pericyte death

To determine whether elevated TNF-α in LVAD patients could suppress Ang-1 expression in pericytes, we incubated cultured pericytes with serum from patients with/without LVADs. Ang-1 gene expression was significantly lower in cultures incubated with serum from LVAD patients compared with HF (0.63 ± 0.09 vs 0.75 ± 0.12 relative quantity (RQ), p < 0.05; Figure 1). This effect in the LVAD group was blunted by TNF-α blockade (0.80 ± 0.09 RQ, p < 0.01), whereas a non-significant increase was observed in the HF group (0.79 ± 0.09, p = NS).

To investigate whether elevated TNF-α in LVAD patients could induce pericyte death, we incubated cultured pericytes with serum from patients with/without LVADs. Pericyte death was significantly higher in cultures treated with serum from LVAD patients compared with HF or OHT patients (10.21 ± 5.90, 5.27 ± 7.03 and 5.76 ± 6.00 AU, respectively; omnibus, p < 0.01; HF vs LVAD, p < 0.05; LVAD vs OHT, p < 0.01; HF vs OHT, p = NS; Figure 2) and this effect in the LVAD group was blunt by TNF-α blockade (6.91 ± 0.09 AU, p < 0.01). No significant difference was observed in the HF or OHT groups with TNF-α blockade (5.99 ± 5.44 and 4.34 ± 5.01 AU, respectively, p = NS). Together, these findings suggest high levels of TNF-α in LVAD patients suppress Ang-1 expression and induce pericyte death.

Table 1 Clinical Characteristics

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<th>HF</th>
<th>LVAD</th>
<th>OHT</th>
<th>p-value</th>
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<td>44</td>
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<td>Age (years)</td>
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<td>58.8 ± 10.7</td>
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<td>Female (%)</td>
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<td>Left ventricular ejection fraction (%)</td>
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<td>59.6 ± 8.4</td>
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<td>Days post-implant (median)</td>
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<td>311.0 ± 1,257.9</td>
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<td>eGFR (ml/min/1.73 m²)</td>
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<td>59.6 ± 24.5</td>
<td>65.8 ± 21.9</td>
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<td>9</td>
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<td>Pulse pressure (mm Hg)</td>
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<td>Anti-platelets (%)</td>
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<td>90</td>
<td>52</td>
<td>&lt;0.001</td>
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ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; eGFR, estimated glomerular filtration rate; HF, heart failure; LVAD, left ventricular assist device; OHT, orthotopic heart transplantation.
Elevated TF protein expression in plasma and endothelial cells from LVAD patients

We measured TF in platelet-poor plasma. TF was significantly higher in the LVAD cohort compared with the HF cohort and trended higher than the OHT cohort (40.92 ± 11.91, 31.74 ± 12.59 and 33.46 ± 7.47 pg/ml, respectively; omnibus, p < 0.01; HF vs LVAD, p < 0.01; LVAD vs OHT, p = 0.118, HF vs OHT, p = NS). To determine the source of the elevated TF in LVAD patients, we analyzed endothelial cells from patients with/without LVADs. TF protein expression in these cells was higher in patients with LVADs compared with HF or OHT patients (6.25 ± 3.94, 1.95 ± 1.37 and 2.51 ± 2.36 AU, respectively; omnibus, p < 0.01; HF vs LVAD, p < 0.05; LVAD vs OHT, p < 0.05; HF vs OHT, p = NS; Figure 3). These findings suggest overexpression of TF in the endothelium may elevate circulating TF levels in LVAD patients.

Elevated TNF-α in plasma from LVAD patients increases endothelial expression of TF and Ang-2

To determine whether increased TNF-α in plasma from LVAD patients could drive expression of TF and Ang-2, we incubated HUVECs with plasma from patients with/without LVADs. Plasma from LVAD patients induced higher TF gene expression than plasma from patients with HF or OHT (5.38 ± 4.2, 1.52 ± 0.96 and 2.03 ± 1.49 RQ, respectively; omnibus, p < 0.01; HF vs LVAD, p < 0.01; LVAD vs OHT, p < 0.05; HF vs OHT, p = NS; Figure 4). In the LVAD cohort, TF expression was significantly reduced with TNF-α blockade (2.88 ± 1.22 RQ, p < 0.05). A non-significant decrease was noted in cultures receiving plasma from patients with HF or OHT (1.18 ± 0.96 and 1.33 ± 0.79 RQ, respectively, p = NS for both). Similarly, the plasma from LVAD patients induced higher Ang-2 gene expression in the HUVECs compared with plasma from patients with HF or OHT (6.19 ± 0.45, 5.02 ± 0.32 and 4.92 ± 0.30 RQ, respectively; omnibus, p < 0.01; HF vs LVAD, p < 0.01; LVAD vs OHT, p < 0.01; HF vs OHT, p = NS). In all cohorts, Ang-2 expression was reduced with TNF-α blockade (4.88 ± 0.34, 3.84 ± 0.49 and 3.83 ± 0.41 RQ, respectively, p < 0.01 for all comparisons). Together, these data suggest elevated TF-α in plasma from LVAD patients induces endothelial expression of both TF and Ang-2.

Elevated TNF-α in serum from LVAD patients induces angiogenesis

To determine whether TNF-α in serum from LVAD patients contributes to endothelial tube formation, we incubated HUVECs on Matrigel with serum from patients with/without LVADs. Serum from LVAD patients induced more tubule formation than serum from patients with HF or OHT (29.52 ± 7.06, 22.00 ± 6.94 and 20.92 ± 8.14 tubes per low-power field, respectively; omnibus, p < 0.01; HF vs LVAD,
p < 0.05; LVAD vs OHT, p < 0.01; HF vs OHT, p = NS; Figure 5A and B). This effect was blunted in LVAD patients by TNF-α blockade (26.42 ± 6.83, p < 0.05), indicating elevated TNF-α in the serum from LVAD patients contributed to tubule formation. No significant difference was observed in the HF or OHT groups in response to TNF-α blockade (22.07 ± 3.69 and 21.50 ± 9.37, p = NS for both).

**Synergic effect of TNF-α and associated molecules on vascular cell motility and survival**

In the Supplementary Material, we confirm the synergic roles of TNF-α, thrombin and Ang-2 in regulating TF expression (Figure S1), angiogenesis (Figure S2), Ang-1 expression (Figure S3) and apoptosis (Figures S4 through S6).

Elevated TNF-α in LVAD patients is strongly associated with increased risk of non-surgical bleeding

To determine whether TNF-α predicts NSB events in LVAD patients, we reviewed the medical records of LVAD patients in this study. Within 1 year of sample collection, 11 patients had NSB, defined as previously reported. Patients who bled within 1 year had significantly higher TNF-α levels than non-bleeders (7.9 ± 1.9 and 5.3 ± 4.9 pg/ml, respectively, p < 0.01). Among LVAD patients with TNF-α levels above the mean, 48% had NSB within 1 year of sample collection (n = 10 of 21) compared with 4% (n = 1 of 23) in patients with TNF-α below the mean (p < 0.01). Among LVAD patients with both TNF-α and Ang-2 above the mean, 67% had NSB within 1 year (n = 6 of 9) compared with 14% (n = 5 of 35) in patients with 1 or both biomarkers below the mean (p < 0.01). The Kaplan–Meier distributions are shown in Figure 6 and Figure S7.

Elevated ROCK activity in LVAD patients

We measured ROCK activity in patients with/without LVADs. As shown in Figure 7, ROCK activity was significantly higher in LVAD patients compared with HF or OHT (2.26 ± 1.77, 1.19 ± 1.01 and 1.02 ± 0.77 RQ, respectively; omnibus, p < 0.01; HF vs LVAD, p < 0.05; LVAD vs OHT, p < 0.05; HF vs OHT, p = NS).

Discussion

In this study we have evaluated the role of TNF-α in promoting vascular destabilization in LVAD patients. We found that high levels of TNF-α induced pericyte apoptosis and suppressed Ang-1 expression. Endothelial TF expression is higher in LVAD patients and high levels of TNF-α induce TF expression in endothelial cells. We previously
found that high levels of thrombin in LVAD patients drive endothelial Ang-2 expression, and the present findings show a contributing mechanism behind thrombin elevation and Ang-1 loss in these patients. Further, high levels of TNF-α in LVAD patients increase angiogenesis in vitro similarly to Ang-2, and TNF-α drives Ang-2 expression. The data suggest there is a synergic effect of TNF-α/Ang-2 on endothelial inflammation and abnormal angiogenesis, as described in tissue culture and mouse models, which likely drives angiodysplasia and vascular instability in LVAD patients. These findings are accompanied by elevation of ROCK activity, a mediator of endothelial inflammation, also associated with vascular instability.

Finally, we found that high TNF-α could increase the risk of NSB while elevation of both TNF-α and Ang-2 together further compounds this risk. Therefore, we propose TNF-α may be a central regulator of LVAD-related angiodysplasia (Figure 8).

Our findings highlight the relationship among inflammatory, coagulation and angiogenic systems and LVAD-related angiodysplasia and help to explain the decrease in Ang-1 we observed. Ang-1 maintains vascular stability, and a low Ang-1/Ang-2 ratio leads to vascular inflammation and is associated with vascular malformations and gastrointestinal angiodysplasia. Histologically, angiodysplasia is associated with endothelial proliferation and pericyte loss. Studies have suggested TNF-α/Ang-2 synergy leads to pericyte apoptosis. Herein we found TNF-α-induced pericyte apoptosis in LVAD patients is associated with suppression of Ang-1, which is blunted by TNF-α blockade.
Studies in tissue culture and animal models have described synergic angiogenic effects of TNF-α/Ang-2. TNF-α primes sprouting endothelial tip cells to receive angiogenic signals and regulates vascular remodeling. Diseases with high TNF-α, such as rheumatoid arthritis, are associated with pathologic angiogenesis, which recedes with anti-TNF-α therapy. Notably, the angiogenic effect of TNF-α appears paradoxical: escalating doses of TNF-α induce more vessel growth until a boundary is reached, where the effect is abolished and toxicity follows. In this study, we have confirmed TNF-α elevation in plasma from LVAD patients and have extended this knowledge by demonstrating that TNF-α induces pericyte apoptosis, suppresses Ang-1, drives angiogenesis, and may increase NSB synergically with Ang-2. Therefore, TNF-α/Ang-2 inhibition may help prevent or treat angiodysplasia in LVAD patients. Indeed, this approach prevents pathologic angiogenesis in mice. Studies have reported on the efficacy of thalidomide in treating LVAD-related NSB. As thalidomide inhibits TNF-α, our findings suggest TNF-α inhibition may explain thalidomide’s efficacy.

High levels of thrombin in LVAD patients contribute to Ang-2 overexpression. Herein we have confirmed TF elevation in LVAD patients and extend this knowledge by demonstrating that TF expression is higher in endothelium of LVAD patients and high levels of TNF-α induce endothelial TF expression. In addition to contact coagulation system activation, as described in our earlier work, and in another study, the synergic effect of TNF-α/thrombin on endothelial TF expression likely creates a feed-forward response in LVAD patients, producing thrombin and leading to overexpression of Ang-2. TNF-α also induces Ang-2 expression independently of thrombin, highlighting the synergy among these factors.

Literature reporting the effect of LVADs on TNF-α is conflicting. In paired analyses (pre- and post-LVAD), myocardial TNF-α decreases after LVAD implantation, whereas LVAD patients have higher plasma levels of TNF-α compared with similar HF patients. We speculate that differences between control groups could explain these discrepancies; patients awaiting LVAD are often very sick and therefore may have high TNF-α levels, which are reduced by LVAD placement, whereas ambulatory HF patients who are functionally similar to LVAD patients may have lower TNF-α. Still, the source of TNF-α in LVAD patients remains unclear. Leukocyte activation by the LVAD, loss of laminar/pulsatile flow and decreased gastrointestinal perfusion could lead to inflammation and TNF-α release.

Our study has several limitations. Although we focused on in-vitro TNF-α inhibition, the effect of inhibiting TNF-α in LVAD patients is not known. It is not possible to account for all confounding variables in human studies, but we sought to minimize confounding with 2 well-matched control groups. We acknowledge that patients are physiologically different from LVAD patients, and the use of tacrolimus and other immunosuppressants in these patients may confound the analyses. Due to the cross-sectional design, changes over time were not addressed. Although TNF-α/Ang-2 may predict future bleeding in LVAD patients, it is not known whether there is a window of time before blood sampling when these markers are associated with bleeding events. Blood flow conditions differ markedly between patients with and without LVADs and...
although evidence linking pulsatility and bleeding exists, the effect on biomarker expression is unknown. Some patients with diseases in which TNF-$\alpha$ is elevated have angiodysplasia, yet others do not. Finally, although the combination of high TNF-$\alpha$/Ang-2 was associated with NSB, the relationship between these markers and angiodysplasia was not directly addressed.

Conclusions

TNF-$\alpha$ is a central regulator of vascular instability and NSB in LVAD patients and likely acts in synergy with Ang-2 and thrombin to augment its effects. Further study is needed to determine whether TNF-$\alpha$ blockade could prevent complications in LVAD patients.

Disclosure statement

V.J. is a scientific consultant for Thoratec/Abbott. N.U. is a scientific consultant for Thoratec/Abbott and Medtronic. The remaining authors have no conflicts of interest to disclose. This work was supported by grants from the National Institutes of Health (HL052233 to J.K.L.), the American Heart Association (17SDG33410656 to C.E.T.) and the International Society for Heart and Lung Transplantation (to C.E.T.).

Supplementary materials

Supplementary materials associated with this article can be found in the online version at www.jhltonline.org/.

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