Serum From Patients With Severe Heart Failure Downregulates eNOS and Is Proapoptotic
Role of Tumor Necrosis Factor-α

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Background—Cytokine activation and endothelial dysfunction are typical phenomena of congestive heart failure (CHF).

We tested the hypothesis that incubating human umbilical vein endothelial cells with serum from patients with CHF will downregulate endothelial constitutive nitric oxide synthase (eNOS) and induce apoptosis.

Methods and Results—We studied 21 patients with severe CHF. Levels of tumor necrosis factor-α (TNF-α) and several neuroendocrine parameters were assessed. eNOS was measured by Western Blot analysis and apoptosis by optical microscopy and flow cytometry. We observed (1) eNOS downregulation (difference versus healthy subjects at 24 hours [P<0.05] and 48 hours [P<0.001]), (2) nuclear morphological changes typical of apoptosis; and (3) a high apoptotic rate with propidium iodide (increasing from 2.1±0.4% to 11.3±1.2% at 48 hours; P<0.001 versus healthy subjects) and annexin V. An anti-human TNF-α antibody did not completely counteract these effects. A strong correlation existed between eNOS downregulation and apoptosis (r=-0.89; P<0.001).

Conclusions—Serum from patients with severe CHF downregulates eNOS expression and increases apoptosis. High levels of TNF-α likely play a role, but they cannot be the only factor responsible. (Circulation. 1999;100:1983-1991.)

Key Words: heart failure ■ tumor necrosis factor ■ endothelium ■ nitric oxide synthase ■ apoptosis

Congestive heart failure (CHF) is a syndrome characterized by neuroendocrine and cytokine activation.1,2 Among cytokines, tumor necrosis factor-α (TNF-α) and its soluble receptors I (sTNF-RI) and II (sTNF-RII) are increased in patients with severe CHF.3–6

In in vitro experiments, high doses of TNF-α induce endothelial dysfunction as a result of the downregulation of endothelial constitutive nitric oxide synthase (eNOS)7 expression and the increase of apoptosis, a mechanism of physiological cell death. Whether or not the negative effects of TNF-α on eNOS and apoptosis also occur in CHF is not known. However, endothelial dysfunction does occur in CHF, as demonstrated in studies in dogs and humans.8,9 This may be due to the activation of several circulating neurohormones.9 Because of the poor correlation between neurohormones and systemic vascular resistances,10 other systems are necessarily involved in this pathological process.11

To evaluate the potential role of TNF-α in endothelial dysfunction, we studied the protein expression of eNOS and the rate of apoptosis of human umbilical vein endothelial cells (HUVECs) after incubation for ≥48 hours with serum from patients with CHF in New York Heart Association (NYHA) class IV. eNOS was evaluated by Western blot analysis and apoptosis by both optical microscopy and flow cytometry.

Methods

Populations Studied

This study was performed at the Cardiology Departments of the Universities of Brescia and Ferrara and at the Heart Failure Unit of the Salvatore Maugeri Foundation, Gussago, Brescia, Italy. A total of 21 male patients with severe CHF (NYHA class IV) and 21 healthy subjects (normal controls) participated in the study. All gave informed consent, and local Ethics Committee approval was obtained. The Table shows the clinical characteristics of the populations studied.

The average age of the patients was 56±9 years. The causes of CHF were coronary artery disease (n=14), idiopathic dilated cardiomyopathy (n=5), or valvular disease (n=2). The mean left ventricular ejection fraction, as assessed by 2D echocardiogram, was 21±5%. Hemodynamic parameters were measured in the postabsorptive state with a Swan-Ganz catheter. Cardiac output was determined by thermodilution with a Gould cardiac output computer (model SP 1445). All patients were treated with angiotensin-
Clinical, Hemodynamic, and Neuroendocrine Characteristics of the Studied Populations

<table>
<thead>
<tr>
<th>NYHA Class IV Patients</th>
<th>Healthy Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=21)</td>
</tr>
<tr>
<td>Age, y</td>
<td>56±9</td>
</tr>
<tr>
<td>Ponderal index, kg/m</td>
<td>39±5</td>
</tr>
<tr>
<td>CHF duration, mo</td>
<td>31±16</td>
</tr>
<tr>
<td>Sodium, mmol/L</td>
<td>135±5</td>
</tr>
<tr>
<td>Ejection fraction, %</td>
<td>21±5</td>
</tr>
<tr>
<td>End-systolic volume, mL</td>
<td>209±100</td>
</tr>
<tr>
<td>End-diastolic volume, mL</td>
<td>262±111</td>
</tr>
<tr>
<td>Cardiac index, L · mm⁻¹ · m⁻²</td>
<td>2±0.5</td>
</tr>
<tr>
<td>Right atrial pressure, mm Hg</td>
<td>16±7</td>
</tr>
<tr>
<td>Mean pulmonary artery pressure, mm Hg</td>
<td>41±10</td>
</tr>
<tr>
<td>Pulmonary capillary wedge pressure, mm Hg</td>
<td>29±8</td>
</tr>
<tr>
<td>Systemic vascular, resistance, dyne · s⁻¹ · cm⁻⁵</td>
<td>1968±453</td>
</tr>
<tr>
<td>Norepinephrine, pg/mL</td>
<td>1004±432</td>
</tr>
<tr>
<td>Renin activity, ng · mL⁻¹ · h⁻¹</td>
<td>34±14</td>
</tr>
<tr>
<td>Aldosterone, pg/mL</td>
<td>505±323</td>
</tr>
<tr>
<td>Atrial natriuretic peptide, pg/mL</td>
<td>445±206</td>
</tr>
<tr>
<td>TNF-α, pg/mL</td>
<td>47.06±14.43</td>
</tr>
<tr>
<td>sTNF-RI, ng/mL</td>
<td>5.11±2.94</td>
</tr>
<tr>
<td>sTNF-RII, ng/mL</td>
<td>9.59±5.46</td>
</tr>
</tbody>
</table>

Data are reported as mean±SD.

Blood Processing

After 30 minutes of supine rest, peripheral venous blood was taken and centrifuged within 1 hour to measure serum electrolytes, norepinephrine (NE), aldosterone, plasma renin activity (RA), atrial natriuretic peptide (ANP), TNF-α, sTNF-RI, and sTNF-RII. All serum samples were stored at −80°C.

Plasma Hormones

Plasma NE levels were measured by high-performance liquid chromatography with electrochemical detection.4,12 Levels of RA, aldosterone, and ANP were measured by radioimmunoassay (DiaSorin, Radin).

TNF-α Immunoactivity

Antigenic TNF-α was determined by a sandwich ELISA with a commercially available kit (Medgenix Diagnostic). The mixture of monoclonal antibodies used does not neutralize TNF-α, which allows the measurement of total circulating TNF-α, even if bound to soluble TNF receptors. The sensitivity of the assay is 3 pg/mL.

sTNF-RI and sTNF-RII

Serum sTNF-RI and sTNF-RII levels were assessed by a sandwich ELISA with a commercially available kit (Amersham). The minimum detectable doses for sTNF-RI and sTNF-RII were 2.5 and 5 pg/mL, respectively.

Cell Culture

HUVECs were isolated from umbilical cords using the method of Jaffe et al.13 No additional growth factor was added to propagate the cultures. HUVECs were characterized by immunofluorescence for von Willebrand factor and by microscopy observation of typical cobblestone morphology.

Between splits 2 and 4, the cells were treated with 40 ng/mL TNF-α (1×10⁶ units/mg) for 6, 12, 24, and 48 hours to establish a positive control group. Moreover, HUVECs were incubated for the same times with 20% serum from either the normal controls or patients with CHF. To test the specific role of TNF-α, we used 1 μg/mL monoclonal anti-human TNF-α antibody (Genzyme).

Nitric Oxide Synthase Analysis

eNOS immunoblotting was performed as previously described.14 Mouse monoclonal anti-human eNOS (Affiniti) and peroxidase-conjugated rabbit anti-mouse IgG (Dako) were used as primary and secondary antibodies, respectively. The specific signal was detected with an enhanced chemiluminescence system (ECL, Amersham) and quantified by densitometry. Each sample was processed 3 times.

Qualitative Assessment of Apoptosis by Optical Microscopy

Optical microscopy assessment was performed by the ApopTag-peroxidase kit and by hematoxylin-eosin and Feulgen staining.

ApopTag-Peroxidase Kit

Cytological samples, fixed in 95% alcohol, were stained with the ApopTag-peroxidase kit (Oncor): the reaction is referred to as TUNEL detection. In this process, residues of digoxigenin nucleotide are catalytically added to DNA by terminal deoxinucleotidal transferase.15

Hematoxylin-Eosin Staining

The cytological slides were placed in Harris hematoxylin (Bio-Optica) for 1 minute and contrasted in bidistilled water. They were converted enzyme inhibitors, diuretics, and digoxin; some were also taking vasodilators, low-dose β-blockers, and positive inotropic agents. Thirteen patients were receiving hepatic hydroxymethylglutaryl coenzyme A reductase inhibitors. Anti-inflammatory drugs were not allowed during the 2 weeks preceding the study. Exclusion criteria included infections; renal failure; pulmonary, thyroid, and collagen vascular diseases; and malignancy.

The normal control group was sex- (male) and age-matched (57±4 years) to the patients. No subjects in this group had any clinical sign of acute or chronic illnesses or any symptoms related to the cardiovascular system.
dehydrated through the increasing alcohol scale and fixed by xylene with Eukitt balsam.16

Feulgen Staining
The cytological slides were treated with HCl at 60°C for 7 to 10 minutes and then placed in Schiff reactive medium (Sigma) for 45 minutes at room temperature, washed in tap water, dehydrated through the decreasing alcohol scale, and fixed by xylene with Eukitt balsam.17

Quantitative Assessment of Apoptosis by Flow Cytometry
Apoptosis was quantified by flow cytometry using a slightly modified version of the method of Nicoletti et al.18 Endothelial cells fixed in 1 mL of cold 70% ethanol were then incubated with a solution of 100 μg/mL propidium iodide (PI) and immediately analyzed. The correct threshold was selected using the apoptotic model in murine thymocytes after 72 hours of culture with dexamethasone 10−4 mol/L. Apoptotic cell nuclei, which were easily distinguishable from debris by the condensation of nuclear chromatin, emitted red fluorescence in red rodamine fluorescence channels 46 to 146. The subdiploid population was detected using the fluorescein isothiocyanate (FITC)–annexin V/PI double-staining method of Vermes et al19; it was then analyzed with a flow cytometer (Cytoron Absolute; Ortho) using ABS software. Dead cells were excluded by setting an appropriate threshold trigger on the low forward light scatter parameter, and nonspecific staining was assessed using FITC-conjugated nonimmune mouse IgG (Coulter).

Statistical Analysis
The significant difference in means of the studied variables was tested by Student’s t test. The correlation between variables was tested by Spearman’s rank or Pearson's correlation methods, depending on the type of distribution of each variable. Multiple linear regression analysis was used to study the possible association between apoptosis and eNOS with TNF-α and hormones. The correlation between variables and the multiple linear regression analysis were performed only on data from patients with CHF. The comparison between patients with CHF and normal controls was analyzed by the linear mixed effects model, with adjustment for multiple comparisons. The tests were considered statistically significant at P<0.05.

Results
The Table shows the clinical characteristics, plasma hormones, and TNF-α data of the populations studied. In patients with CHF, the cardiac index was severely reduced (2±0.5 L·min⁻¹·(m²)⁻¹), the right atrial and pulmonary pressures were high (16±7 and 41±10 mm Hg, respectively), and the systemic vascular resistances (1968±453 dyne·s⁻¹·cm⁻⁵) and serum levels of sodium were lowered (135±5 mmol/L). Mean values for RA and aldosterone were 34±14 ng·mL⁻¹·h⁻¹ and 505±323 pg/mL, respectively. Plasma concentrations of NE and ANP were 3.4 and 17 times greater, respectively, than those of the normal controls. The mean values of antigenic TNF-α in patients with CHF and in normal controls were 47.1±14.4 and 21.6±4.1 pg/mL, respectively (P<0.001). The same patterns were recorded for the soluble receptors of the cytokine (P<0.001 for both sTNF-R1 and sTNF-RII versus normal controls).

eNOS
Figures 1 and 2, respectively, show a representative Western blot of eNOS and the mean data for each group obtained after incubation with exogenous TNF-α 40 ng/mL (positive control) and serum from normal controls and patients with CHF, both with and without the anti-human TNF-α antibody. As expected, the incubation of the HUVECs with TNF-α resulted in a downregulation of eNOS expression: after 6 hours, a 23% reduction versus normal control was observed (P<0.05), which reached 85% after 48 hours (P<0.001). The addition of the anti-human TNF-α antibody completely counteracted this decrease.

In normal controls, the incubation of HUVECs had no effect on eNOS, whereas in patients with CHF, it resulted in a time-dependent downregulation of the protein expression (P<0.05 and P<0.001 versus normal controls after 24 and 48 hours, respectively). The TNF-α antibody partially counteracted the inhibitory effect of the serum from patients with CHF.

Multiple linear regression analysis was performed with the variables from patients with CHF that significantly correlated with eNOS expression. After step-wise selection, TNF-α levels showed a mild correlation to the reduction of eNOS expression (r=0.51; P<0.05).

Qualitative Assessment of Apoptosis by Optical Microscopy
The apoptotic pattern, as observed with optical microscopy, is shown in Figure 3. HUVECs from normal controls did not undergo relevant changes (A through C), whereas those from patients with CHF showed changes in nuclear profile and alterations of chromatin structure (D through F). The immunocytochemical ApopTag-peroxidase staining (brown positive nuclei) showed the increase of the DNA 3'OH terminals that was generated by the internucleosomal rupture, which precedes the detachment of the apoptotic bodies. With this method, in the normal controls, a positive nucleus could be...
occasionally detected in some fields; conversely, in patients with CHF, \( \geq 1 \) positive nucleus was detectable in each field (D). This finding was also confirmed by the hematoxylin-eosin and Feulgen stains (E and F). The most frequent alteration was nuclear membrane expansion (sometimes ovoidal), which was associated with hyperchromatic zones; these nuclei appeared as pyknotic nuclei later evolving in apoptotic bodies.

**Quantitative Assessment of Apoptosis by Flow Cytometry**

Figure 4 shows apoptosis over 6, 12, 24, and 48 hours as measured by flow cytometry using a solution of PI as the fluorochrome after membrane ethanol permeabilization. The top panels show representative cytfluorometric recordings of HUVECs in positive and normal controls and patients with CHF. As expected, a proportional induction of apoptosis occurred over time, which reached a maximum of 33.7±2.6% at 48 hours. Obviously, the induced apoptosis was completely counteracted by adding the anti-human TNF-α antibody (\( P<0.001 \)) (Figure 4, lower left). The lower right panels show the mean data obtained from the incubation of HUVECs with the serum from either normal controls or patients with CHF, with and without the anti-human TNF-α antibody. Over 48 hours, the percentage of apoptotic cells in normal controls increased from 1.8±0.8% to 4.2±0.5%. In patients with CHF, a higher rate of apoptosis existed when compared with normal controls; this higher rate could be seen at 6 hours, and it increased from 2.1±0.4% to 11.3±1.2% at 48 hours (\( P<0.001 \) at both 24 and 48 hours). The addition of the anti-human TNF-α antibody to the serum from patients with CHF reduced, but did not counteract, its apoptotic effect (\( P<0.001 \) at both 24 and 48 hours versus CHF without antibody).

To better quantify apoptosis, we also performed flow cytometry using annexin V/PI double staining (Figure 5). Annexin V binds with a high affinity to phosphatidylserine, which is normally located in the inner part of the cell membrane. In the early apoptotic states, phosphatidylserine translocates from the inner to the outer layer of the cell membrane, thus allowing its binding to annexin V. PI used without ethanol acts as a cellular viability marker. Therefore, annexin V reveals the early apoptotic processes of still-viable cells, whereas PI, which binds to DNA as a consequence...
of end-stage apoptosis or ongoing necrosis, reveals signs of membrane permeability impairment. The upper panel of Figure 5 shows 3 dot-plots of HUVECs relevant to positive controls, normal controls, and patients with CHF. The dot-plot of the positive controls clearly identified 3 different clusters of cells: (1) viable ones that were not stained by either of the 2 markers, (2) apoptotic ones that were positively stained only by annexin V, and (3) end-stage apoptotic or necrotic cells that were stained by both markers. The dot-plot from the normal controls did not show relevant positivity to annexin V or PI, whereas incubation with serum from patients with CHF showed a similar cell distribution to that of the positive controls. The mean data are shown in the lower panels and resemble the data presented in Figure 4, although annexin V revealed higher apoptosis than PI at each evaluation time.

**Correlation Analyses**

A strong negative correlation exited between the downregulation of eNOS expression and apoptosis \( (r = -0.89; P < 0.001) \), suggesting a link between the 2 phenomena. In addition, multiple linear regression analysis performed on patients with CHF, including all the parameters studied, showed significant correlation with apoptosis. After step-wise selection, only TNF-\( \alpha \) blood levels were significantly related to apoptosis \( (r = 0.56; P < 0.05) \). A strong correlation also existed between TNF-\( \alpha \) and its receptors \( (r = 0.76; P < 0.05 \text{ for both sTNF-RI and sTNF-RII}) \) and between the 2 receptors \( (r = 0.87; P < 0.01) \). Conversely, no correlation was found among apoptosis, neurohormones, or any of the clinical parameters measured.

**Discussion**

We investigated some aspects of the endothelial function of patients with CHF by applying an original experimental
approach. We tested the serum of patients with CHF on the in vitro function of HUVECs. Because we used the serum of patients as the “real” clinical mean, we avoided the artificial use of single or multiple exogenous inducers of endothelial dysfunction, as occurs in the classical in vitro/ex vivo experiments. Moreover, by determining HUVEC eNOS expression and apoptosis, we overcame the limitations of the indirect evaluation of endothelial dysfunction that are typical of clinical studies. HUVECs (human origin) resemble a homologous milieu, although they are not strictly indicative of endothelial cells in other systemic blood vessels.

Our data show that serum from patients with severe CHF downregulates eNOS expression and increases apoptosis, indirectly suggesting endothelial dysfunction. Because no correlation existed between neurohormones and the 2 end points relevant to the endothelial function measured in the current study, we anticipate a possible role of another system: the system of cytokines and, in particular, TNF-α. Although our findings cannot be fully extrapolated to in vivo clinical conditions, they do represent the consequences of abnormal interaction between the bloodstream of patients with CHF and human endothelium. Obviously, our data cannot demonstrate any organ/tissue dysfunction because endothelium is only 1 of the involved components.

Serum from Patients With CHF Downregulates the Expression of eNOS. Is This a TNF-α–Mediated Process?

The endothelium regulates vascular tone by producing vasodilating and vasoconstrictive substances. In normal vessels, acetylcholine induces NO synthesis by activating eNOS. Conversely, in patients with CHF, acetylcholine results in a blunted vasodilating response, whereas nitric oxide–donor administration exerts a vasodilating response similar to that observed in normal controls, suggesting the integrity of the vascular muscle cell. One explanation for this apparent paradox is that eNOS expression is impaired in CHF. A recent study in dogs with severe CHF indicates that the impaired response to acetylcholine is due to reduced eNOS expression, confirming that the 2 phenomena are closely linked.8

Cytokines, when used in vitro, inhibit eNOS expression.21 Our data, which were obtained by directly administering TNF-α to HUVECs expressing TNF receptors, confirm this finding. Thus, the direct administration of TNF-α to HUVECs can be referred to as an alternative cytotoxicity assay because it resembles the physiopathological conditions.

The incubation of the HUVECs with serum from patients with CHF also resulted in a time-dependent downregulation of eNOS expression.
of the protein expression, showing a weak correlation only with TNF-α. This weak correlation could be due to the small sample size and to the difference in sensitivity between the Western blot technique and the immunoenzymetric test. Moreover, TNF-α does not completely account for eNOS downregulation, because the addition of the anti-human TNF-α antibody partially counteracts the effect of CHF serum on eNOS after 48 hours. Because the effects of TNF-α on the expression and activity of eNOS in vitro are enhanced by interferon γ and interleukin 1β, it is possible that this cytokine mixture, present in the blood of patients with CHF, is responsible for eNOS downregulation. Interestingly, the incubation of the HUVECs with 40 pg/mL TNF-α, which corresponds to the mean plasma concentration of our patients, only had a minor effect on eNOS expression (data not shown). This suggests that the combination of TNF-α with other factors is responsible for eNOS downregulation.

Our data must also be considered in view of the actual treatment the patients were receiving, including angiotensin-converting enzyme inhibitors and hepatic hydroxymethylglutaryl coenzyme A reductase inhibitors, which increase the expression and activity of eNOS through a bradykinin-mediated and a post-transcriptional mechanism, respectively.

Serum from Patients with CHF Induces Apoptosis. Is This a TNF-α–Mediated Process?

Apoptosis is a mechanism by which the cell actively participates in its own death by activating enzymes that induce morphological and structural changes on cell components (eg, nucleus, membrane, etc). An abnormal apoptotic rate is possibly involved in the pathogenesis of several cardiovascular diseases, including atherosclerosis, restenosis, conduction-system defects, and several pathological features of CHF, such as myocardial and endothelial dysfunction. Measurement of apoptosis, however, is not an easy process; therefore, a method should be used, particularly in studies on human tissues, as we have done.

The initial description of apoptosis was based on morphological features; therefore, we examined the HUVECs by optical microscopy using the ApopTag-peroxidase kit and the hematoxylin-eosin and Feulgen stains. Typical apoptotic nuclei were shown in the HUVECs treated with serum from patients with CHF. We also found evidence of specific DNA fragmentation (in the form of fragments of 180 bp or multiples) in all HUVECs treated with serum from patients with CHF (data not shown).

Both these techniques are subject to criticism because they are mainly qualitative. Therefore, to properly quantify apoptosis, we also used flow cytometry with 2 different fluoro-
chromes: PI and annexin V-FITC. Flow cytometry allows the detection of events occurring in thousands of cells when compared with microscopy, which can only explore limited fields. Because PI detects apoptosis only in the advanced stages, when the loss of fragmented DNA is already an ongoing process, we also used annexin V to quantify the early phases of apoptosis. In addition, we combined annexin V with PI but without ethanol, thus using PI as a dye-exclusion test to establish the integrity of cell membranes. The calculation of the percentage of apoptosis did not include the cells with marked membrane alterations because, at end-stage apoptosis, membrane permeability changes in apoptotic and necrotic cells are indistinguishable.

Our data confirm that, in vitro, the endothelium has a physiological rate of apoptosis that is not influenced by cytokines; they also show that serum from patients with CHF is proapoptotic. More than 30 inducers of apoptosis have been identified, including neurohormones, cytokines, and newly discovered genes and second messengers, thus making it difficult to identify the primum movens.

We tested the hypothesis that TNF-α plays a crucial role in the complex biochemistry of apoptosis signaling. This cytokine induces its cytotoxic effects by binding to specific receptors present in almost all cells. TNF-RI has a cytoplasmic signaling motif called the death domain, which induces caspases activation, resulting in inactivation of several proteins controlling different functions. All these changes induce the morphological characteristics that differentiate apoptosis from necrosis. Interestingly, we found a 5-fold increase in sTNF-RI, suggesting that TNF-α interacted with this receptor.

TNF-α may increase the inducible type of nitric oxide synthase (iNOS) expression, and the resulting peroxinitrite would induce apoptosis. However, this sequence of events is expected in bovine aortic endothelial cells but not in HUVECs. We did not observe any induction of iNOS (data not shown), thus ruling out the possibility of TNF-α-induced, high-output nitric oxide production in human endothelial cells.

Although TNF-α is one of the main regulators of endothelial cell life/death equilibrium, other factors may play a major role in the maintenance of this ratio. Among these, NFκB, whose activation can be stimulated by TNF-α itself under specific conditions, increases the resistance to the apoptotic stimuli; angiotensin II, by a different modulation of the receptors AT₁ and AT₂, can have a pro- or antiapoptotic effect; and Bcl-2 family members can also promote or inhibit apoptosis induced by certain triggers.

Conclusions

Our results support the hypothesis that the circulating blood of patients with CHF causes endothelial dysfunction. TNF-α likely plays a role, but it is not the only responsible factor.

Acknowledgments

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References


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