Cytokines Are Not a Requisite Part of the Pathophysiology Leading to Cardiac Decompensation (44461)

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Abstract. **An increase in circulating levels of proinflammatory cytokines has been proposed as an important pathogenic factor contributing to cardiac injury during chronic heart failure. To determine whether plasma levels of the cytokines tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) increase during pacing-induced heart failure, we paced the hearts of seven dogs at 210 beats/min for 3 weeks and at 240 beats/min for an additional week to induce severe clinical signs of cardiac decompensation. Hemodynamic measurements and blood samples from the aorta and coronary sinus (CS) were taken at control, at 3 weeks, and in end-stage failure. Decompensated heart failure occurred at 29 ± 1.8 days, when left ventricular (LV) end-diastolic pressure was 25 ± 1.3 mmHg, LV systolic pressure was 92 ± 4 mmHg, mean arterial pressure was 77 ± 3 mmHg, and dP/dt_{max} was 1219 ± 73 (all $P < 0.05$ vs control). Arterial concentration of IL-6 was 12 ± 4.0 U/ml at control, 11 ± 2.7 U/ml at 3 weeks, and 10 ± 1.7 U/ml in end-stage failure (NS). At the same time points, IL-6 in CS plasma was 12 ± 3.5, 13 ± 2.8 and 11 ± 2.4 U/ml, respectively (NS vs control and vs arterial concentrations). TNF-α did not reach detectable concentrations in arterial or CS blood at any time. TNF-α and IL-6 concentrations did not increase in arterial blood, were not released in the CS from the heart during the development of pacing-induced heart failure, and can not universally be implicated in the pathogenesis of all forms of cardiac dysfunction. Our findings are consistent with other data from patients in which severe heart failure was not associated with increased levels of circulating cytokines.**

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Cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6), are a class of potent endogenous peptides released by macrophages, leukocytes, and endothelial cells in response to injury (1–3). Able to exert numerous actions on different tissues at high concentrations, cytokines can affect cardiovascular function by direct inhibition of myocardial contractility (4) or by uncoupling myocardial β-adrenergic receptors (5). In particular, the direct negative inotropic effect seems to be mediated through a nitric oxide synthase (NOS), IL-1 and -6 and TNF-α activate constitutive NOS (4) or induce the expression of inducible NOS (iNOS) (6) with consequent generation of excessive amounts of NO, which is a potent myocardial depressant when present at high, nonphysiological concentrations (7, 8). The findings of elevated circulating levels of TNF-α (9) and nitric oxide metabolites (10) in patients with severe chronic heart failure led to the hypothesis that proinflammatory cytokines contribute to the malignant progression of heart failure by causing myocardial dysfunction (11). We have recently found that pacing-induced heart failure is not characterized by an increase, but rather by a decrease in cardiac production of NO metabolites (12), inconsistent with elevated cytokines and NO production. In patients with decompensated heart failure, the increase in plasma TNF-α correlates with the level of cachexia rather than with ejection fraction or severity of New York Heart Association (NYHA) functional

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class, as found by Levine *et al.* (9) and McMurray *et al.* (13). Munger *et al.* reported that only IL-6 was elevated in mild or moderate heart failure, likely representing a chronic marker of inflammation associated with myocardial damage (14). On the basis of these studies, it is evident that cardiac impairment is not necessarily associated with elevated levels of TNF-α or interleukins, and the role of cytokines in the malignant progression of chronic heart failure remains hypothetical. Therefore, the aim of the present study was to determine whether the evolution of pacing-induced heart failure in dogs is characterized by changes in plasma concentrations and in cardiac release of TNF-α and IL-6.

Materials and Methods

Surgical Procedure and Instrumentation. Male dogs (n = 7) weighing 25–27 kg were sedated with acepromazine maleate (1 mg/kg, i.m.), anesthetized with sodium pentobarbital (25 mg/kg, i.v.), and ventilated with room air. A thoracotomy was performed in the left fifth intercostal space. Catheters (Tygon, Cardiovascular Instruments, Boston, MA) were placed in the descending thoracic aorta and left atrial appendage for pressure measurements. A third catheter was inserted in the coronary sinus with the tip leading away from the right atrium. During the surgery, a blood sample was taken from the coronary sinus cathether and immediately measured on a pH/blood gas analyzer (Instrumentation Laboratory, Lexington, MA). If the PO_2 was >25 mmHg, the catheter was repositioned and tied into place. A solid-state pressure gauge (P6.5, Konigsberg Instruments, Inc., Pasadena, CA) was inserted in the left ventricle through the apex. A Doppler flow transducer (Craig Hartley, Houston, TX) was placed around the left circumflex coronary artery. A human, screw-type, unipolar myocardial pacing lead was placed on the left ventricle (LV). Wires and catheters were run subcutaneously to the intracapsular region, the chest was closed in layers, and the pneumothorax was reduced. Amoxicillin, 400 mg/day, was given for 6 days after surgery, and the dogs were allowed to recover fully. After 10 days, dogs were trained to lie quietly on the laboratory table. The protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the New York Medical College and conform to the guiding principles for the care and use of laboratory animals published by the National Institutes of Health.

Hemodynamic Recordings. The aortic catheter was attached to a P23ID strain-gauge transducer for measurement of aortic pressure. Left ventricular pressure (LVP) was measured using the solid-state pressure gauge. The first derivative of LVP, LV dP/dt, was obtained using an operational amplifier (National Semiconductor LM 324, Newark, NJ), and triangular wave signals with known slope were substituted for the pressure signals to calibrate the differentiator directly. Coronary blood flow (CBF) was measured with a pulsed Doppler flowmeter (Model 100, Triton Technology, La Jolla, CA). All signals were recorded on a 14-channel tape recorder (Bell and Howell 3700B, New Brunswick, NJ), and played back on an eight-channel direct-writing oscillograph (Gould RS 3800, Cleveland, OH). Mean values of aortic pressure and coronary flow were obtained by filtering the respective signals at 2 Hz. Heart rate was measured using a cardiotachometer (model 9857B, Beckman Instruments, Fullerton, CA) from the LVP pulse interval. The heart was paced using an external pacemaker (Pace Medical, Waltham, MA) carried by the dog in a vest.

Blood Gas Measurements. Blood samples from the aorta and coronary sinus were collected into plastic syringes treated with heparin and immediately stored on ice. Special care was taken to withdraw blood slowly from the CS to avoid potential contamination of the sample with right atrial blood. The pH, PO_2, and PCO_2 were measured with a blood gas analyzer (Instrumentation Laboratory, Mod. 1306).

Measurements of TNF-α and IL-6 in Plasma. After measurements of blood gases, blood was transferred to 14-ml centrifuge tubes and spun at 1000g at 4°C for 15 min. Plasma was then collected in separate plastic tubes and frozen at –80°. For cytokine measurements, plasma samples were first allowed to thaw at room temperature. TNF-α concentration was measured by enzyme-linked immunoadsorbent assay (Cytoscreen Immunoassay Kit, Biosource International, Camarillo, CA). We have used this assay previously (15, 16). Briefly, standards consisting of recombinant rat TNF-α were used at concentrations of 1—1000 pg/ml. Samples, including standards of known TNF-α concentration and unknowns, were pipetted into wells coated with antibody specific for TNF-α. A second biotinylated antibody, which binds to a second site on the TNF-α antigen, was then added. Samples were then incubated for 1.5 hr at room temperature and aspirated to remove any excess unbound biotinylated antibody. The enzyme streptavidin peroxidase, which binds to the TNF-α-bound biotinylated antibody, was then added. This solution acts on bound enzyme to produce color. Absorbance of colored products was measured by spectrophotometric analysis at a wavelength of 450 nm. The absorbance of standards was plotted versus concentration, and plasma TNF-α concentrations were calculated (pg/ml) on the basis of the standard curve. The sensitivity of this assay to canine TNF was tested by a serial dilution of plasma samples, up to a ratio 1:32, from three previous experiments in dogs in which high levels of circulating cytokines were induced by lipopolysaccharide injection (16). The assay results were sensitive and linear over a range of TNF-α concentrations from 3 to 120 pg/ml.

IL-6 concentrations were determined by bioactivity assay (17). Briefly, plasma samples were heat-inactivated at 56°C for 30 min. Bioactivity was measured by monitoring the ability to induce proliferation of murine B9 hybridoma cells. The cell cultures were incubated for 92–96 hr, and the growth of B9 cells was then quantitated using the uptake of MTT (3-[4,5 dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) and measured at 570/690-nm wavelengths using the EL312 Biotek microplate reader (Biotek, Norcross,

GA). The World Health Organization reference standard 89/548 was included in every assay. IL-6 concentration was measured in units per milliliter. We have used these methods previously in dogs (16).

Induction of Heart Failure and Protocol. Hemodynamic measurement and paired blood samples from the aorta and CS were first taken at control, in the presence of stable heart rate and blood pressure. Heart failure was induced by pacing the heart at 210 beats/min for 3 weeks, then the pacing rate was increased to 240 beats/min until overt heart failure was observed. During this time, 400 mg of amoxicillin were given if febrile episodes occurred. Antibiotics do not interfere with the synthesis of TNF-α and IL-6 or with the assays used in our study (2). Hemodynamic measurements and blood samples were taken again at 3 weeks of pacing and in end-stage failure. We have previously shown that cardiac decompensation occurs after the third week of pacing (12). End-stage failure was defined as the time when left ventricular end diastolic pressure (LVEDP) reached 25 mmHg, and clinical signs of severe decompensation were observed. At this time, the dog was sacrificed. All measurements were performed with the pacer turned off and the heart in spontaneous rhythm.

Calculations and Statistical Analysis. Data are presented as mean ± SEM. Statistical analysis was performed using commercially available software (Sigma Stat 2.0). Changes in hemodynamics and cytokine concentrations were tested using one-way ANOVA for repeated measurements followed by Bonferroni's test. For all the statistical analysis, significance was accepted at $P < 0.05$.

Results

End-stage heart failure occurred at 29 ± 1.8 days, when LV end diastolic pressure was 25 ± 1.3 mmHg, and severe clinical signs such as dyspnea, ascites, pale mucosae, and lethargy were present.

Hemodynamics. Changes at 3 weeks and in end-stage failure in LV end diastolic pressure, mean arterial pressure, LV systolic pressure, LV dP/dt_{max}, and heart rate are listed in Table I. All these hemodynamic values changed significantly at 3 weeks, and there was a further increase in LVEDP and a decrease in mean arterial pressure (MAP) in end-stage heart failure. Mean left circumflex coronary blood flow was 27 ± 4.0 ml/min at control, 28 ± 3.9 ml/min at 3 weeks, and 28 ± 5.0 ml/min in end-stage failure (NS). At 29 ± 1.8 days, cardiac decompensation and congestive heart failure were associated with a fall of arterial PO_2 to 74 ± 6 mmHg, from a control value of 93 ± 2 mmHg ($P < 0.05$). PO_2 in CS blood was 25 ± 1.8 mmHg at control, 22 ± 1.4 mmHg at 3 weeks, and 22 ± 2.0 mmHg in end-stage failure (NS).

TNF-α and IL-6. TNF-α was never detectable in arterial or coronary sinus blood samples, either at control or during the progression of heart failure.

IL-6 concentrations in arterial and CS blood, at control, 3 weeks, and end-stage heart failure, are presented in Figure 1. There were no significant changes at any time.

Discussion

The present study demonstrates that plasma concentration or cardiac release of TNF-α and IL-6 are not significantly altered during the decompensation of pacing-induced heart failure. After 3 weeks of pacing, all the hemodynamic values were significantly altered, and overt heart failure occurred at 29 ± 1.8 days, when LV end diastolic pressure reached 25 mmHg, arterial PO_2 fell significantly, and dogs presented ascites, dyspnea, lethargy, and pale mucosa. Despite the occurrence of these clinical signs of congestive heart failure, plasma TNF-α concentrations were undetectable; IL-6 was detectable, but did not change significantly. Therefore, our findings do not support the hypothesis that cytokines play an important role in the progression of all forms of chronic heart failure toward cardiac decompensation. To our knowledge, this is the first study in which cytokines have been measured in animals with pacing-induced heart failure.

The absence of changes in cytokine levels could be interpreted as the consequence of profound differences between chronic heart failure in humans and pacing-induced heart failure in dogs. Several reports, in fact, clearly showed that patients with severe heart failure present high levels of circulating cytokines (9, 13, 18, 19). A careful evaluation of those clinical studies, however, addresses the apparent discrepancy between our findings and the results in humans. Levine *et al.* (9) studied a group of 33 patients, all belonging

Table I. Changes in Hemodynamics During the Evolution of Pacing-Induced Heart Failure

	Pacing Time (days)		
	0 (Control)	21	29 ± 1.6 (End stage)
LVEDP, mmHg	5.1 ± 0.8	14.0 ± 1.6[a]	25.0 ± 1.3[b]
LVSP, mmHg	132.0 ± 4.0	103.0 ± 5.4[a]	92.2 ± 3.0
MAP, mmHg	108.0 ± 3.2	90.0 ± 3.8[a]	77.0 ± 2.5[b]
dP/dt_{max}, mmHg/sec	2860 ± 88	1475 ± 38[a]	1219 ± 73[a]
HR, bpm	84.0 ± 8.2	105.0 ± 3.1	125.0 ± 5.6[a]

Note. LVEDP indicates left ventricular end diastolic pressure; LVSP, left ventricular systolic pressure; MAP, mean arterial pressure; dP/dt_{max}, maximum value of the first derivative of LV pressure; HR, heart rate. $n = 7$, data are mean ± SEM. [a]$P < 0.05$ vs control. [b]$P < 0.05$ vs 21 days.

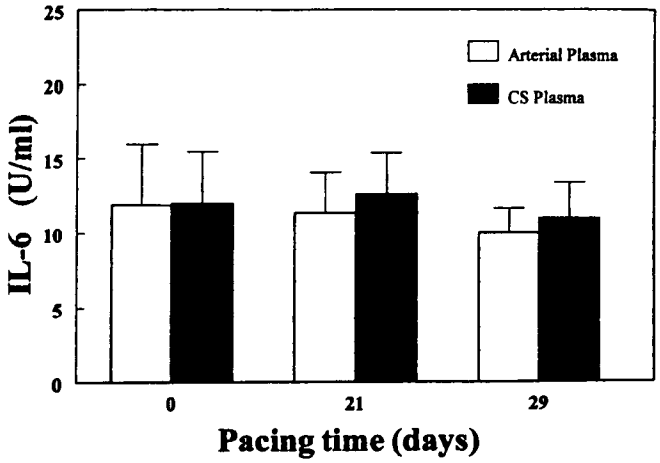

Figure 1. Concentrations of IL-6 in arterial and CS plasma during the evolutoin of pacing-induced heart failure. There were no statistically significant differences vs control and between arterial and CS concentrations. Data are mean ± SEM. $n = 7$.

to NYHA functional class III and IV. Only 19 patients had TNF-α serum concentrations >39U/ml (i.e., exceeding by more than 2 SD the mean level seen in the control group). In 12 of the remaining patients, equally characterized by severely impaired cardiovascular function, TNF-α concentration was 0 or as low as that measured in control subjects. Those authors found that high concentrations of TNF-α were associated with signs of cachexia (i.e., systemic decay characterized by reduced body fat, skeletal muscle wasting, and marked alterations in biochemical parameters). To further support the association between cachexia and TNF-α, McMurray *et al.* (13) found that, in non-cachectic patients with heart failure in NYHA class III and IV, TNF-α was undetectable, whereas it was elevated, between 15 and 200 pg/ml, in only half of the patients with cachexia. A marked increase in TNF-α levels, during the most advanced states of cardiac cachexia syndrome, is not surprising, since this cytokine is a marker and probably one of the pathogenic factors leading to cachexia (20) and it is also increased in other debilitating diseases, such as full-blown acquired immunodeficiency syndrome (21), or during the processes of defense against cancer (22) and systemic infections (23). It is conceivable therefore that elevated levels of TNF-α represent a nonspecific component of terminal heart failure, when other pathogenic mechanisms have already caused cardiac impairment. This particular evolution does not necessarily occur in all patients. It is true that TNF-α can depress cardiac function directly *in vivo,* but this typically occurs during malignant septic shock from gram-negative bacteria. In a study on 79 patients with septicemia and/or meningococcal meningitis, Waage *et al.* (24) found that TNF-α was detectable in 18 cases and necessarily associated with fatal outcome only when the concentration was between 440 and 100,000 U/mL. It should be noted that Levine *et al.* (9) detected a TNF-α concentration between 400 and 600 U/ml in only 2 of the 33 patients with chronic heart failure. We have shown previously that administration of bacterial lipopolysaccharides in dogs causes a dramatic increase in circulating TNF-α and IL-6, a six-fold increase in plasma NO metabolites and a four-fold increase in NO generated by inducible NOS in coronary microvessels (16). This is the typical picture of septic shock in which the increase in cytokines precedes NO overproduction, has devastating consequences on cardiovascular function, and leads to a rapid and fatal progression of the disease. Such a rapid and dramatic progression does not occur during chronic heart failure. *In vitro,* TNF-α and IL-6 exert a clear, negative inotropic effect only at concentrations ranging between 900 and 3200 U/ml (4), by far higher than those measured in plasma of patients with chronic heart failure (9). IL-6 may even be beneficial by preventing the transition to heart failure during cardiac mechanical stress, as suggested by a very recent study in mice lacking the gp130-cytokine receptor (25). Finally, a major source of confusion in the interpretation of the role of cytokines in chronic heart failure is derived from different methods adopted to measure cytokine concentration and different levels found by the authors in normal subjects. Mohler *et al.* (26) measured a TNF-α average concentration of 5.69 pg/ml in blood from patients with heart failure, 6 times higher than in control subjects. The lack of an increase in TNF-α reported by several clinical studies on chronic heart failure was attributed by Mohler *et al.* (26) to the poor sensitivity of the assays used. However, the same assay used by Mohler *et al.* was used by Wu *et al.* to measure plasma TNF-α in patients with gastric cancer (27). In that study, the levels found in healthy control subjects were between 2 and 10 pg/ml and Wu *et al.* used a cutoff of 10 pg/ml to define significant and pathological increases in TNF-α. A cutoff of 5–10 pg/ml was also customary in other reports (22, 28). Ferrari *et al.* (19) reported a mean TNF-α concentration of 33.5 pg/ml in patients with chronic heart failure in NYHA functional class IV and of 14 pg/ml in normal subjects. Based on these data, it can be concluded that TNF-α concentrations ranging from 0 to 15 pg/ml may not be clinically significant.

Haywood *et al.* have shown intense TNF-α immunostaining in endothelial and vascular smooth muscle cells of intramyocardial blood vessels in patients with dilated cardiomyopathy (11). However, the present study could not demonstrate significant differences in concentrations of TNF-α and IL-6 between arterial and coronary sinus blood during the progression of heart failure, suggesting that pacing-induced heart failure is not characterized by high levels of cytokines in cardiac tissue. The negative results cannot be explained simply on the basis of profound differences between pacing-induced cardiomyopathy and human disease since a clinical study failed to demonstrate a difference in arterial versus coronary sinus concentration of cytokines either in normal subjects or in patients with heart failure of various etiology, including dilated cardiomyopathy (14). Those patients presented a left ventricular ejection fraction of 20%. The only change found in that study, as well as in the study by Mohler *et al.* (26) was an increase in systemic

levels of IL-6, yet this cytokine is notoriously induced in many cell types by almost every noxious stimulus and is readily found in the peripheral circulation (29). The increased IL-6 in patients with heart failure was interpreted as a chronic marker of inflammation associated with myocardial damage (14). It is also important to consider that a very high percentage of patients with severe heart failure undergo invasive diagnostic and therapeutic procedures with exposure to repetitive inflammatory stimuli. Catheter-related bacteremia develops in ≈ 50,000 patients yearly in the United States, and more than 90% of these infections are associated with central venous or arterial catheters (30). On the other hand, we did not interfere with the natural evolution of pacing-induced heart failure; however, we administered amoxicillin to cure infections.

In our study, we only measured IL-6 and TNF-α concentrations in arterial and coronary sinus blood since it is with respect to these two factors that other authors proposed an important role of cytokines in the genesis of chronic heart failure (9–11, 13, 14). IL-1 is the most potent known inducer for IL-6 (31,32). Therefore IL-1 was most likely not increased in our study, given the unchanged levels of IL-6 in arterial and coronary sinus blood during the progression of pacing-induced heart failure. We did not determine the gene expression of TNF-α and IL-6 in cardiac tissue; however, this is transient and declines after 90 min in the case of TNF-α and after 3–4 hr in the case of IL-6, even though the cytokines remain detectable in blood (32). Moreover, locally produced IL-6 immediately egresses to the circulating pool (29, 32), and eventual increases in TNF-α in the dogs used in our study would have been reflected by increases in IL-6 (33). For all these reasons, the measure of TNF-α and IL-6 in blood is sufficient to provide a critical evaluation of cytokine production.

In summary, pacing-induced heart failure is not characterized by changes in circulating levels of TNF-α and IL-6 nor in release of these cytokines in coronary sinus blood. Our results do not support the hypothesis that increased production of cytokines is required for cardiac injury during the development of chronic heart failure.

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