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Quantification of proinflammatory cytokines in the urine of congestive heart failure patients. Its relationship with plasma levels

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Abstract

Aims: Proinflammatory cytokines are important mediators for the development of heart failure and increased plasma levels of these cytokines have been reported in patients with this condition. The purpose of the study was to investigate whether urine, a non-invasively obtained biological sample, was an appropriate medium in which to measure the concentration of tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) in patients in the advanced stages of the disease. *Methods and results:* Thirty consecutive patients who had severe congestive heart failure (NYHA classes III and IV) and 30 matched healthy control subjects were enrolled. Plasma and the first urine of the day were collected and TNF-α and IL-6 were quantitatively analyzed by enzyme-linked immunosorbent assays. For every subject there were no differences in the amount of cytokine determined in plasma and urine. Both urine and plasma levels of IL-6 and TNF-α were greater in heart failure patients than in controls. *Conclusion:* Our results show that plasmatic and urinary levels of proinflammatory cytokines did not differ significantly. Thus, urine may be a good milieu in which to study these cytokines and may have diagnostic, prognostic and therapeutic implications.

Keywords: Congestive heart failure; Urine; Cytokines; Interleukin-6; Tumor necrosis factor-alpha

1. Introduction

The mechanisms that explain the progressive dysfunction of the failing myocardium remain largely unknown. An imbalance of immune mediators together with an inappropriate enhancement of proinflammatory cytokines or even reduction of anti-proinflammatory mediators have been recently recognized to play an important role in the pathophysiology of cardiac diseases, not only in acute disorders but also in chronic conditions such as congestive heart failure (CHF) [1,2]. From in vitro and in vivo studies, the proinflammatory cytokines, interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) have been proposed as important mediators in the development of heart failure [3,4] but their precise pathophysiological role is still unclear.

Cytokines are extremely pleiotropic proteins, synthesized and secreted after stimulation of different cell types, that exert their biological function through the interaction with specific cell membrane receptors in an autocrine or paracrine fashion. Activated cells present in cardiac tissue [5] such as macrophages, endothelial cells and myocytes are producers of TNF-α, and TNF-α receptors of type 1 and type 2 have been identified in cardiac myocytes [6]. In addition, TNF-α mediates a negative inotropic effect on the myocardium by several mechanisms [7] and apoptosis in cardiac myocytes [8] and also contributes to progressive left ventricular remodeling [9]. Recently, its implication in the increased oxidative stress [10] observed in patients with dilated

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cardiomyopathy or mild cardiac insufficiency has been demonstrated.

IL-6 is expressed in cells present in the cardiac parenchyma [11] such as activated macrophages and endothelial cells. An interesting point is that the site of production of both proinflammatory cytokines, IL-6 and TNF-α, in CHF patients does not seem to be restricted to cardiac tissue [12]. Despite the known pathophysiological actions of TNF-α and IL-6 in experimental models, its clinical importance in CHF is still unclear, though these cytokines have developed a potential significance. In fact, blood levels of these cytokines have been found to be moderately elevated in CHF patients [13]. Recent studies reveal that several treatments for cardiac diseases including angiotensin-converting enzyme inhibitors [14] and other experimental treatments, such as positive inotropic substances [15] or anti-proinflammatory cytokines [16], were downregulators of these mediators. Other drugs that act directly against the cytokines, as a TNF-α receptor analogue, are being tested in clinical trials [17].

Nothing is known about cytokine levels in the urine of patients with CHF despite the interest in their role as potential mediators of disease progression and even though the urine levels of cytokines have demonstrated a high diagnostic specificity in other pathologies [18] and the sample is non-invasively obtained. Therefore, the aim of this study was to determine the urinary excretion of IL-6 and TNF-α in patients with severe CHF and healthy subjects and to compare them with the plasma levels, in order to explore if the observed plasma alteration of proinflammatory cytokines is also evident in urine.

2. Methods

2.1. Patients

Sixty subjects were included in this study. Thirty of them were consecutive patients who had severe heart failure established on the basis of medical history, electrocardiogram, chest X-ray and echo-Doppler study [19]. The cause of heart failure was dilated cardiomyopathy ($n=11$), ischemic cardiomyopathy ($n=9$), hypertension ($n=5$) and valvular heart disease ($n=5$). The patients were functionally defined according to New York Heart Association in classes III (15 subjects) and IV (15 subjects) and were receiving standard medical treatment following the guidelines of the American Heart Association [20] and the European Society of Cardiology [21]. Gender distribution was 20 males and 10 females and the mean age 67 ± 11 years. Subjects with acute coronary syndromes and those with acute and chronic liver disease, rheumatoid arthritis, chronic infections, pulmonary diseases, malignancy or urinary tract pathologies were excluded. All patients were on stable medical therapy for at least 1 month before enrollment in the study and none were receiving anti-inflammatory drugs. The other 30 subjects were healthy controls matched in age and gender and without any history of cardiac disease, hypertension, diabetes mellitus or hyperlipidemia. The Investigation Review Boards of our Institutions approved the study protocol and all participants gave written consent before entering the study.

2.2. Blood and urine samples

As there is a high correlation between 24-h cytokine concentrations and spot samples [22], patients and controls were asked to collect the first urine of the day. The same morning, in a fasted state, peripheral blood was collected by venipuncture into pyrogen-free vacuum tubes containing EDTA as anticoagulant. Urine and anticoagulated blood tubes were immediately centrifuged (15 min at $400\times g$) and the sediment-free urine and plasma samples were obtained. Both plasma and urine samples were aliquoted and stored at -80 °C until further analysis and thereafter were only thawed once.

2.3. Cytokine measurement

Plasma and urine concentrations of IL-6 and TNF-α were measured in duplicate by specific commercial sandwich enzyme-linked immunosorbent assays (ELISA) following manufacturer's recommendations (Quantikine, R&D systems Inc, Minneapolis, MN). The tests were quantified at 450 nm in a dual wavelength microplate reader (Sunrise, TECAN, Austria) with the help of Magellan software (version 2.5 TECAN, Austria). The ELISAs were suitable for the quantitative determinations of cytokines in both plasma and urine and analyte concentrations found in samples were in the range of linearity of each assay. Results were expressed as pg/ml.

2.4. Statistical analysis

SPSS 7.0 package (SPSS Inc, Illinois, USA) was used for statistical analysis. Student's *t*-test assuming equal variances was used to assess differences in continuous variables for unpaired data (patients and controls) and the paired data (plasma vs. urine determinations). Data is presented as the mean ± S.E.M. (standard error of the mean). A P value lower than 0.05 was considered as statistically significant.

3. Results

3.1. Plasma and urine concentrations of IL-6

As can be seen in Fig. 1, in CHF patients the mean ± S.E.M. plasma concentration of IL-6 was

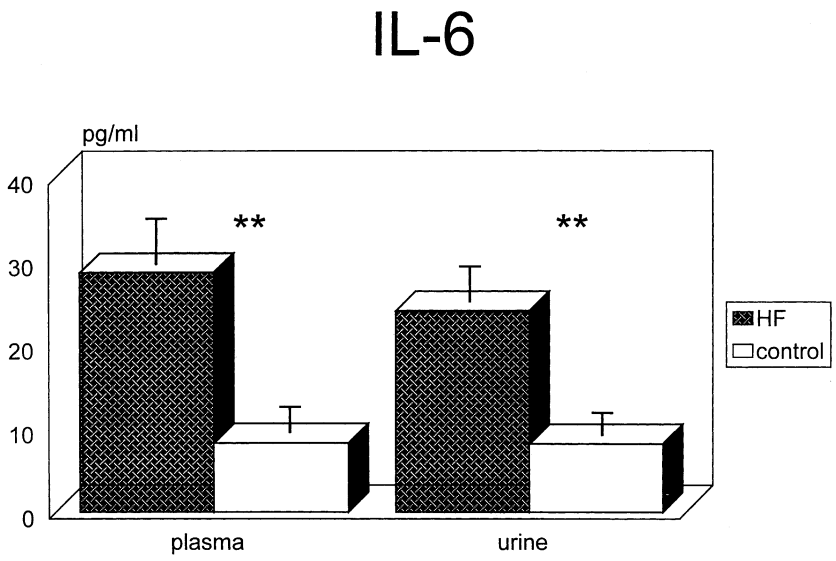

Fig. 1. Mean ± S.E.M. plasma and urine concentrations of IL-6 in CHF patients ($n=30$) and healthy controls ($n=30$). **$P<0.001$.

28.6 ± 5.4 pg/ml while in the control group it was 8.2 ± 2.1. This difference was statistically significant ($P<0.001$). In the same figure the mean urine values of IL-6 in the CHF group and in the control group, being 24.0 ± 3.8 and 8.1 ± 0.8, respectively, are shown. In urine, the elevation observed in CHF patients, when compared with healthy controls, was also significant ($P<0.001$).

When we compared for every subject and in every group the plasma and the urine concentrations of IL-6, we did not find any statistically significant differences.

3.2. Plasma and urine concentrations of TNF-α

Fig. 2 shows the mean ± S.E.M. values of TNF-α in plasma and urine samples for both groups. In CHF patients we found that plasma and urine concentrations of TNF-α were 5.1 ± 1.5 and 3.9 ± 1.3, respectively. In the control group plasma and urine levels of TNF-α were 2.0 ± 0.5 and 1.8 ± 0.3, respectively. When we compared with normal subjects we found that the plasma TNF-α elevation observed in CHF patients was significant ($P<0.05$) while the urine elevation seen did not reach statistical significance.

For every subject and in every group plasma and urine concentrations of this cytokine did not differ significantly.

4. Discussion

CHF is characterized by complicated cardiorenal, hemodynamic and neurohormonal alterations [23]. In an

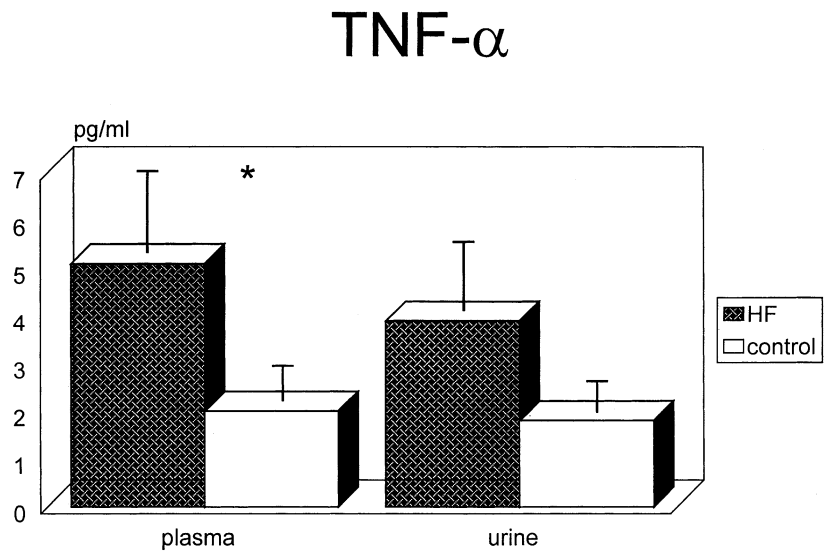

Fig. 2. Mean ± S.E.M. plasma and urine concentrations of TNF-α in CHF patients ($n=30$) and healthy controls ($n=30$). *$P<0.05$.

attempt to delineate a biochemical mechanism for the cachexia that occurs in patients with advanced heart failure, it was discovered that patients with advanced heart failure expressed elevated levels of TNF-α in their peripheral circulation [24]. In the ensuing years since this observation, there has been increasing evidence that TNF-α might also directly contribute to the progression of heart failure [25,26] by virtue of the direct toxic effects that this molecule exerts on both the heart [27] and the circulation [28].

Recent studies suggest that IL-6 may be physiopathologically linked to impaired function in advanced heart failure by influencing critical determinants of cardiac performance systemically and by direct effects on the heart [29]. One of the signals that up-regulates IL-6 synthesis is TNF-α, which has been implicated in the development of ventricular hypertrophy and depression of myocardial contractility through stimulation of its receptor which is expressed in cardiac myocytes [30].

Our study's objective was to analyze the urinary excretion and the plasma levels of proinflammatory cytokines in patients with severe CHF, in order to confirm if the previously described enhanced plasma concentrations of IL-6 and TNF-α were also detectable in urine. As cytokines are glycoproteins of relatively low molecular mass, rarely more than 20 kDa, and cationic molecules smaller than this molecular mass are free filtered through glomeruli as easily as water [31], we have speculated that proinflammatory cytokines could be present in the urine of patients without urinary tract pathologies. In the present study, we have demonstrated for the first time that proinflammatory cytokines could be measured by commercial immunoassay in the urine of CHF patients, and that their concentrations are significantly greater than those obtained from healthy controls.

Our results therefore confirm the findings from several other reports demonstrating elevated levels of proinflammatory cytokines in the plasma of CHF patients [32,33]. We have shown a significant increase in the plasma levels of IL-6 and TNF-α in patients with heart failure in NYHA classes III and IV. The same findings can be observed in the urine of these patients when compared with healthy matched controls, but only IL-6 elevation in the urine of CHF patients attains statistical significance. Several reasons may explain why TNF-α levels in the urine of heart failure patients were elevated, like IL-6, but were not statistically significant. It is possible that high levels of interfering factors could cause unusual results in some cases. In fact, the presence of soluble receptors for these cytokines, usually present in biological samples, could interfere with the measurement of their ligands in the samples. Another drawback of this study was that an important number of determinations in urine were slightly above the minimum detectable concentration of TNF-α, around the limits of the test's precision, and this could affect the statistical significance.

A limitation of the study concerning the determinations of proinflammatory cytokines in urine is that these molecules are mainly produced and consumed locally in an autocrine or paracrine manner. If, as some authors have proposed, the failing myocardium is the principal synthesizer and receptor of proinflammatory cytokines [11,19,26], then the levels may not be as elevated in peripheral blood as in cardiac tissue and therefore the leakage into the urine could be so difficult to measure, that very sensitive analytical procedures would be needed. In this regard, it is worth mentioning that there are some contradictory data demonstrating that the elevation of cytokine concentration in the myocardial tissue is a late event, and it is preceded by elevated plasma levels of proinflammatory cytokines [34].

A common limitation with these kind of studies is that patients, as expected, are receiving conventional therapy for their disease, and it is known that several kinds of drugs can reduce the blood levels of these proinflammatory cytokines. In fact, angiotensin-converting enzyme inhibitors and glycosides have been shown to decrease plasma levels of both IL-6 and TNF-α because some of them are inhibitors of proinflammatory cytokine synthesis. However, this study confirms that a high degree of immune activation persists in heart failure patients even during standard therapy, and can be detected in plasma and urine. On the other hand, it is known that certain substances can diminish plasma levels of important pathophysiological mediators, such as myocardial steroids [35], while expression at the heart can still be elevated. This suggests that the relationship between tissue, plasma and urinary levels of proinflammatory cytokines, as well as the relationship between cytokine reduction in plasma and the efficacy of heart failure drugs requires further investigation.

In conclusion, this study expands on previous publications that have described elevated levels of IL-6 and TNF-α in heart failure, by demonstrating that urinary levels of proinflammatory cytokines increase in patients in relation to heart failure symptomatology, and that these levels are similar to those obtained in plasma. These findings are consistent with the point of view that overexpression of proinflammatory cytokines in patients with CHF and deteriorated functional status may be one of several different maladaptive biochemical mechanisms that change the patient status from asymptomatic ventricular dysfunction to symptomatic heart failure [23]. If confirmed, urine may be a good medium to study the cytokine imbalance observed in CHF patients. These new data may have diagnostic implications and can provide the basis for the development of new suitable methods with prognostic value that would need to be clarified in specifically designed studies and might

also enable us to evaluate the efficacy of therapeutic interventions.

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