Clinical research

The pro-apoptotic serum activity is an independent mortality predictor of patients with heart failure

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Aim Systemic inflammation with elevated serum levels of circulating pro-inflammatory cytokines is a major determinant of prognosis in heart failure (HF). Since serum of patients with HF induces apoptosis of endothelial cells (EC), we aimed to determine whether the pro-apoptotic activity in the serum may predict prognosis of patients with HF.

Methods and results We measured the pro-apoptotic activity in the serum of 48 patients with HF of different aetiology by an ex vivo cell culture assay and subsequently monitored these patients for the single endpoint all-cause mortality. During follow-up, 16 patients died and 11 patients received a heart transplant. Survivors had a lower pro-apoptotic serum activity (P = 0.007). By univariate analysis, pro-apoptotic serum activity, NYHA class, pro-BNP, low blood pressure, and creatinine levels were significantly associated with mortality. In a multivariable stepwise Cox-regression model, the pro-apoptotic serum activity (adjusted hazard ratio, HR = 1.85 per %, P = 0.008), elevated pro-BNP levels (HR = 9.35 per log[pro-BNP], P = 0.001), and low blood pressure (HR = 0.96 per mmHg, P = 0.041) remained as independent predictors of death.

Conclusion In this exploratory study, the pro-apoptotic serum capacity is independently associated with a worse prognosis in patients with HF, suggesting that the assessment of serum-induced EC apoptosis could provide an integrative estimate of the deleterious effects of various pro-inflammatory cytokines and other cytotoxic factors in HF.

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KEYWORDS
Heart failure; Prognosis; Apoptosis; Serum marker; Endothelial cells

Introduction

Current understanding of the pathophysiology of advanced heart failure (HF) implicates neurohumoral acti-
ou studies have demonstrated that the serum of patients with HF is pro-apoptotic for EC.\textsuperscript{5–7} We, therefore, hypothesised that the pro-apoptotic activity in the serum of patients with HF may quantitatively integrate the cytotoxic insult to EC by neurohumoral activation and, as a functional read-out of the entire cascade of pro-inflammatory cytokines, may affect disease progression in patients with advanced HF.

Methods

Patients

Forty-eight Patients with HF were recruited between June 1998 and August 2000 via our outpatient HF clinic at the occasion of routine visits or when referred for elective, invasive examinations in order to proceed with an evaluation for heart transplantation. At the time of inclusion, patients were on a titrated medical regimen (see Table 1). Patients with a history of myocardial infarction (MI) within three months before blood sampling as well as patients with decompensated renal insufficiency (defined by serum creatinine levels $\geq 2$ mg/dl) or concomitant infectious or primary pulmonary disease were excluded. Ischaemic aetiology was defined by the presence of significant coronary artery disease documented by coronary angiography and a history of previous MI. Venous blood samples were drawn into 10 ml serum tubes without additives, and, following centrifugation, serum aliquots were frozen at $-80$ °C. All patients included in this study gave written, informed consent. The study protocol was approved by the Ethics Committee of the Johann Wolfgang Goethe University of Frankfurt.

Definition of endpoint

Patients were monitored via our outpatient and heart transplant programme at standard intervals over a minimum follow-up of 30 months (median follow-up for survivors 1254 days). All-cause mortality was the single endpoint. Patients, who received a heart transplant during follow-up, were considered as survivors for the time period from inclusion to the date of transplantation with no further follow-up beyond this point of time (“censored alive”).

Ex vivo pro-apoptotic activity assay

As previously reported in detail,\textsuperscript{6} the systemic pro-apoptotic activity was assessed by an ex vivo cell culture assay. In brief, human umbilical venous endothelial cells (HUVEC, Cell Systems/Clonetics, Solingen, Germany) were grown in endothelial basal medium (EBM) supplemented with hydrocortisone, bovine brain extract, gentamicin, amphotericin B, epidermal growth factor, and 10% foetal calf serum until the third passage before experiments were performed. For quantification of the pro-apoptotic activity of patient serum, cell culture medium was removed, cells were washed, and subsequently incubated with a modified medium, which contained 10% patient serum. Following 18 h of incubation, nuclei were stained and the pro-apoptotic activity of the serum was quantified as the percentage of apoptotic nuclei out of the total number of cells. Two independent, blinded investigators assessed features of nuclear morphology characteristic for apoptotic cells by fluorescence microscopy as described previously.\textsuperscript{4} The reproducibility of these measurements was determined in a subset of individuals ($n = 20$ patients and healthy subjects) by the comparative analysis of two aliquots of the same serum sample in two individual HUVEC passages from the same lot. The intra-sample inter-HUVEC correlation of these measurements was $r = 0.86$, $P < 0.001$. For the inter-observer variability between two blinded investigators, the correlation was $r = 0.77$, $P < 0.001$ in $n = 63$ individuals (patients and healthy subjects).

Cytokine and pro-BNP assays

TNF\textsubscript{a} and soluble TNF receptor 1 (sTNF R1) serum concentrations were measured by enzyme-linked immunosorbent assays (ELISA, R & D Systems, Wiesbaden). NT-pro-brain natriuretic peptide (pro-BNP) was determined by ELISA (Roche Diagnostics).

Statistics

Data are expressed as mean $\pm$ SD or as indicated. Cumulative survival was univariately evaluated by Kaplan–Meier analysis (log-rank test). Hazard ratios (HR) were calculated by univariate Cox regression analysis. Factors with a strong univariate significance indicated by a $P$-value below 0.005 were stepwise included in a multivariable Cox regression model in order to adjust factors for their interdependency. Individual parameters

### Table 1 Clinical characteristics and medical treatment of 48 CHF patients at inclusion

| Age (years) | 57 $\pm$ 1 |
| Gender (male/female) | 37/11 |
| NYHA class (II/III/IV) | 20/21/7 |
| Aetiology (ischaemic/non-ischaemic) | 22/26 |
| LVEF (%) | 25 $\pm$ 1 |
| LVEDD (mm) | 65 $\pm$ 1 |
| Intraventricular conduction delay/QR duration (ms) | 23 (48)/128 $\pm$ 5 |
| Rhythm (Sinus/A. fib./Pacemaker) | 34/12/2 |
| Medical treatment | |
| ACE-Inhibitor/AT\textsubscript{1} Receptor Blocker | 48 (100) |
| $\beta$-Blocker | 20 (42) |
| Spironolactone | 11 (23) |
| Diuretics | 43 (90) |
| Digitalis | 36 (75) |
| Amiodarone | 18 (38) |
| Statin | 11 (23) |
| Implanted cardioverter/defibrillator | 11 (23) |
| Surgery (LV reduction/assist devices) | 1 (2)/2 (4) |
| Serum parameters | |
| pro-BNP (pg/ml) | 3666 $\pm$ 595 |
| TNF\textsubscript{a} (pg/ml) | 3.6 $\pm$ 0.7 |
| sTNF-R1 (pg/ml) | 1670 $\pm$ 112 |
| Apoptosis (%) | 4.3 $\pm$ 0.2 |
| Haemodynamics | |
| Mean arterial blood pressure MAP (mmHg) | 85 $\pm$ 2 |

*Numbers of patients (% of all patients) or mean $\pm$ SEM.*
that failed to reach a significance level of $P < 0.05$ when comparatively analysed in this model were omitted from further multivariable analysis. The assumption of proportional hazards was validated by defining a time-dependent covariate as a function of time ($T$) and individual covariates. The assumption of linearity was tested by fractional polynomials. No correction has been made for multiple hypothesis testing since this, because of the small sample and endpoint size, was an exploratory study searching for parameters requiring confirmation in subsequent adequately powered studies. Statistical significance was assumed, if the null hypothesis could be rejected at $P = 0.05$. All analyses were performed with SPSS software (version 11.5).

Results

Clinical and serological characteristics of patients at baseline

Baseline clinical characteristics, the pro-apoptotic serum activity as well as cytokine and pro-BNP levels of the 48 patients are summarised in Table 1. The pro-apoptotic serum activity, as well as serum pro-BNP and cytokine levels, were correlated with disease severity (data not shown).

Clinical outcome

Sixteen patients died during follow-up at a median time period of 289 days (interquartile range 676) after inclusion. Eleven patients received a heart transplant after a median of 217 days (interquartile range 262) following inclusion, at which time these patients were censored as survivors.

In the subgroup of patients without heart transplants, baseline serological and clinical parameters were as follows: the pro-apoptotic serum activity at inclusion was $4.97 \pm 1.11\%$ in patients with a lethal outcome vs. $3.97 \pm 0.98\%$ in those surviving without heart transplantation. Patients, who subsequently died, had pro-BNP levels of $5825 \pm 4259$ pg/ml and sTNF-R1 levels of $2105 \pm 875$ pg/ml, whereas in patients, who survived without heart transplantation, pro-BNP was $1712 \pm 3465$ pg/ml and sTNF-R1 was $1238 \pm 455$ pg/ml. Serum levels of TNF$_\alpha$ were $3.6 \pm 1.2$ pg/ml in patients, who died, and $4.1 \pm 6.9$ pg/ml in patients surviving without heart transplantation.

According to clinical symptoms, patients, who died during follow-up, had serum creatinine levels of $1.4 \pm 0.4$ mg/ml and a serum sodium concentration of $138 \pm 4$ mM vs. $1.1 \pm 0.2$ mg/ml and $140 \pm 3$ mM, respectively, in patients surviving without heart transplantation. The echocardiographic left ventricular ejection fraction (LVEF) was $22 \pm 8\%$ in those, who subsequently died, compared to $28 \pm 7\%$ in patients, who survived without receiving a heart transplant. In this subgroup, patients who died, had a median mean arterial blood pressure (MAP) of $78 \pm 12$ mmHg, whereas the MAP was $93 \pm 10$ mmHg at inclusion in those, who survived without heart transplantation.

Predictors of mortality

Dichotomisation of the patient population according to the pro-apoptotic serum activity revealed a significantly higher mortality of patients with pro-apoptotic activity above the median value as assessed by Kaplan–Meier analysis (Fig. 1).

In order to test the predictive value of various risk factors in our study population for the occurrence of death within 30 months, we analysed the data by univariate Cox regression. The pro-apoptotic serum activity significantly influenced survival in HF patients, in addition to established prognostic factors such as the severity of clinical symptoms according to NYHA classes, levels of pro-BNP, blood pressure, and serum creatinine concentrations (Table 2). Age, echocardiographic LVEF, tachycardia (defined as a heart rate above 90 bpm), hyponatraemia (defined as a serum sodium concentration below 135 mM), and hyperuricaemia (serum uric acid above 9.5 mg/ml) were weakly associated with a worse outcome, but did not reach statistical significance to predict mortality in the tested cohort.

Independent predictors of mortality

To determine independent predictors of mortality among these variables, we used a stepwise multivariable model of regression analysis including every factor with a univariately significant influence on mortality. Among these, high pro-apoptotic serum activity, elevated BNP levels, and low blood pressure remained the only independent predictors of death in patients with HF (Table 3).

By ROC curve analysis, both the sensitivity and specificity of the pro-apoptotic serum activity to predict mortality (AUC 0.756) were comparable to those for pro-BNP serum levels (AUC 0.787, Fig. 2).
Discussion

Our data demonstrates for the first time that the pro-apoptotic serum activity predicts mortality in patients with HF. Importantly, the pro-apoptotic serum activity remained a significant predictor of mortality following stepwise multivariable regression including various clinical, serological, and routine laboratory characteristics of patients with HF. In this model, the predictive capacity of the pro-apoptotic serum activity was comparable to the strong serological prognosticator of mortality in HF (pro-BNP serum levels) as well as to the leading clinical symptom of the low output syndrome, hypotensive blood pressure.

The prognostic relevance of the pro-apoptotic serum activity as a quantitative marker of endothelial activation indicates the potential pathophysiological contribution of systemic vascular inflammatory processes to the progression of HF beyond haemodynamic alterations and independent of the aetiology of cardiac dysfunction. While the extent and composition of the pro-inflammatory, oxidative and general cytotoxic burden in HF may relate to both cardiac and peripheral vascular dysfunction, the ex vivo assay used in our study clearly displays the capacity of these factors to induce apoptosis in EC. The independent prognostic impact of this pro-apoptotic activity towards EC underscores an important pathophysiological link, through which the pro-apoptotic serum activity...
load exerts its deleterious action during HF progression. However, as in vitro read-out systems to study the induction of cardiomyocyte apoptosis by human serum are not available, we cannot rule out that sera of patients with HF may likewise exert a pro-apoptotic activity towards cardiomyocytes, and that such an effect would also influence prognosis in HF. Further studies are necessary to analyse the individual pathophysiological role of various tissue activation by inflammatory stimuli in HF.

Our data may also highlight the significance of an integrative measure of pro-apoptotic cytotoxicity for the progression of heart failure beyond the assessment of individual factors with a known capacity to induce cell death. Serum levels of TNFα, which was among the first serological factors to be recognised as deleterious during the course of heart failure,5,6 were not significantly different in survivors compared to patients, who died during follow-up. Only extreme elevations of TNFα levels (above the 80%-tile or >3.75 pg/ml) were significantly associated with an increased risk for mortality (P = 0.015 by univariate Cox regression analysis). The limited predictive value of TNFα levels is in accordance with previous data, which showed that blocking antibodies against TNFα did not completely abolish apoptosis induction by the serum of heart failure patients but only reduced it to a minor extent.5,6 Moreover, serum levels of C-reactive protein (CRP), which is considered a surrogate marker of systemic pro-inflammatory activity, had no significant influence on mortality in our population. Taken together, these data suggest that the combined action of more than one cytotoxic factor, rather than TNFα, contributes to apoptosis induction in EC by the serum of patients with heart failure.

A major limitation of the present study relates to the rather small sample size of patients. However, by univariate analysis, the previously established predictive parameters of HF mortality pro-BNP10 and creatinine levels as well as advanced NYHA class and lower blood pressure showed a highly significant association with reduced survival, thus confirming that the patient population studied was indeed representative of HF characteristics. Despite the rather small sample size of the patient cohort, the study size was sufficiently powered to indicate the independent predictive value of the pro-apoptotic activity in a multivariable regression model including other established predictors of mortality in HF. Moreover, ROC curve analysis disclosed the usefulness of measuring pro-apoptotic serum activity to predict mortality in patients with HF with sensitivity and specificity similar to measuring serum pro-BNP levels, the prototypic prognostic serum marker in patients with HF. Nevertheless, the development of a routinely accessible, standardised measurement procedure for the assessment of the pro-apoptotic serum activity based on the experimental design used in the present study will be necessary to independently validate the prognostic relevance within the scope of larger trials.

Another limitation of the present study is the rather infrequent use of β-blockers among our patients, since in the year 1998, when enrolment of patients into this study began, the use of β-blockers in patients with advanced heart failure was less well-established. However, we have previously reported that the β-blocker carvedilol, but not metoprolol protects EC against apoptosis in vitro and in vivo in patients with heart failure.6 Thus, future determinations of the pro-apoptotic serum activity are mandatory to confirm the survival impact of this parameter at the current standard medication.

In conclusion, the pro-apoptotic serum activity as an integrative measure of the combined cytotoxic serum load represents a strong independent predictor of mortality in patients with HF, thus further underscoring the importance of systemic inflammatory responses in the pathophysiology of HF.

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