Follistatin-Like 1 in Chronic Systolic Heart Failure
A Marker of Left Ventricular Remodeling

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Background—Follistatin-like 1 (FSTL1) is an extracellular glycoprotein found in human serum. Recent work suggests that FSTL1 is secreted in response to ischemic injuries and that its overexpression is protective in the heart and vasculature.

Methods and Results—We examined serum FSTL1 levels in patients with chronic heart failure with left ventricular (LV) ejection fraction <40% (n=86). The sample was separated into three tertiles of patients with low, medium, and high FSTL1 levels. Serum FSTL1 was increased 56% above age- and sex-matched healthy controls. Diabetes mellitus, brain natriuretic peptide level, left atrial size, LV posterior wall thickness, LV end-diastolic diameter, and LV mass were significant determinants of FSTL1 serum levels by bivariate analysis. After controlling for significant covariates, FSTL1 levels predicted LV hypertrophy (as measured by LV mass index) by multivariate linear regression analysis (P<0.001). Unadjusted survival analysis demonstrated increased mortality in patients with increasing FSTL1 levels (P=0.09). After adjusting for significant parameters, patients with increased FSTL1 remained at the highest risk of death (hazard ratio, 1.028; 95% CI, 0.98 to 1.78; P=0.26). To determine whether elevated FSTL1 levels may be derived from the myocardium, FSTL1 protein expression was measured in explanted failing (n=18) and nonfailing (n=7) human hearts. LV failing hearts showed 2.5-fold higher FSTL1 protein levels over nonfailing control hearts (P<0.05).

Conclusions—Elevated serum FSTL1 in patients with heart failure was associated with LV hypertrophy. Further studies on the role of FSTL1 as a biomarker in chronic systolic heart failure are warranted. (Circ Heart Fail. 2011; 4:621-627.)

Key Words: follistatin-like 1 protein human ▪ heart failure systolic ▪ hypertrophy left ventricular

Follistatin-like 1 (FSTL1), also referred to as transforming growth factor-β-stimulated clone 36, or TSC36, is an extracellular glycoprotein that has a follistatin-like domain.1 Members of the follistatin family regulate the transforming growth factor-β superfamily proteins through their ability to function as binding partners and antagonize the binding of ligands to the receptors. Recently, it was shown that disco-interacting protein 2 homolog A functions as an FSTL1 receptor.2 Although FSTL1 is reported to suppress cancer growth and invasion in animal models3 and modulate inflammation and allograft survival,4,5 little is known about its function in cardiovascular disease. We previously found that FSTL1 is upregulated and secreted in cardiovascular injury models where it promotes myocyte survival and ischemia-induced revascularization.6,7

Clinical Perspective on p 627

Widera et al8 found that FSTL1 levels are increased in acute coronary syndrome and associated with all-cause mortality. In patients with end-stage heart failure (HF) on left ventricular assist device (LVAD) therapy, cardiac FSTL1 gene expression was increased and subsequently declined when LV function improved.9 Collectively, these findings suggest that FSTL1 can serve as a cardiac tissue-derived marker of cardiovascular disease. To explore the role of FSTL1 in the pathogenesis of human systolic HF, we measured serum FSTL1 levels in patients with chronic LV systolic dysfunction to determine its relationship with measures of LV cardiac remodeling. Additionally, we measured FSTL1 protein levels from the LV of patients with end-stage failure.
HF undergoing heart transplantation. More precisely, we sought to test the following hypotheses: (1) Serum FSTL1 levels are elevated in the patients with HF compared to controls; (2) FSTL1 protein expression is increased in explanted failing LV human tissue; and (3) in patients with chronic systolic HF, there is an association between serum FSTL1 levels and clinical markers of cardiac remodeling.

**Methods**

**Subjects**

Eighty-six patients with chronic HF and LV ejection fraction <40% were recruited between 2001 and 2004 from an ambulatory HF clinic at Boston Medical Center. Data were recorded at enrollment, and the analysis reflects the measurements of all 86 patients. A medical history was obtained to document etiology, symptoms by New York Heart Association functional class, and coexisting diseases. Routine laboratory test results (eg, electrolytes, blood count) and concomitant cardiac medications were recorded. Body mass index was calculated as the ratio of weight to height squared. Twenty-one healthy volunteers of similar age and sex distribution were used for comparison. The Boston Medical Center Institutional Review Board approved the study. All subjects gave written, informed consent. LV systolic dysfunction etiology was defined as follows: (1) ischemic (history of myocardial infarction [by ECG and positive troponin], results of a positive noninvasive stress test, or cardiac catheterization); (2) hypertensive (documented history of pharmacologically treated hypertension); (3) idiopathic (having no identifiable cause of the cardiomyopathy); and (4) other, including valvular, alcohol-induced, and familial etiologies.

**Echocardiography**

Two-dimensional and Doppler echocardiography were performed at baseline as previously described using the Vingmed Vivid Five System (GE Healthcare; Milwaukee, WI) with a 2.5-MHz phased-array transducer. Echocardiograms were performed and analyzed in a blinded manner. Measurements of systolic and diastolic chamber dimensions and wall thickness were obtained from 2D imaging according to the recommendations of the American Society of Echocardiography. The standard cubic formula was used to calculate LV mass.

**Biomarker Analysis**

Blood samples were collected and serum decanted. Samples were stored at −80°C. Brain natriuretic peptide (BNP) levels were measured by the ADVIA Centaur assay (Siemens Healthcare Diagnostics; Deerfield, IL). Routine laboratory analysis was performed at the Boston Medical Center clinical laboratory.

**Serum FSTL1 Measurements**

Serum FSTL1 levels were determined by quantitative Western blotting. Serum samples were added to 10-fold volumes of blue loading buffer (Cell Signaling Technology Inc; Danvers, MA), and separated on SDS-PAGE (Lonza Group Ltd; Basel, Switzerland). Proteins were transferred onto PVDF (GE Healthcare UK Ltd; Chalfont St Giles, Buckinghamshire, UK) and probed with the antihuman FSTL1 antibody (1/2000) (R&D Systems Inc; Minneapolis, MN) followed by incubation with the antigoat IgG HRP secondary antibody (1/1000) (Santa Cruz Biotechnology Inc; Santa Cruz, CA). The ECL (GE Healthcare UK) Western blotting detection reagents and analysis system were used for the detection of the protein signal. The signal intensities were standardized by recombinant human FSTL1 protein (R&D Systems) and quantified using Image J software (National Institutes of Health; Bethesda, MD). At a molecular weight of 50 kDa, the FSTL1 band intensity was measured by densitometry and after adjusting to a standard curve created from 3 different doses of recombinant human FSTL1 protein, was expressed as arbitrary units.

**Human Myocardial Tissue Procurement**

Failing LV tissues were obtained from patients with end-stage nonischemic diluted cardiomyopathy (n = 9) and ischemic cardiomyopathy (n = 9) at the time of cardiac transplantation. For comparison, nonfailing (NF) human LV tissues were obtained from organ donors (n = 7) with no known history of cardiac disease that could not be transplanted for technical reasons. All subjects or organ donor family members gave written consent for tissue donation. The study was reviewed and approved by the Ethical Committee of the University Medical Center Hamburg-Eppendorf (Az 532/116/9.7.1991).

**Tissue Western Blot Analysis**

Membranes were blocked with 5% (weight/volume) dried milk in 100 mmol/L Tris (pH 7.5), 0.1% (volume/volume) Tween 20, and 150 mmol/L NaCl (TBST) for 1 hour before overnight incubation at 4°C with the primary antibodies. Primary antibodies were used against α-actinin (1/1000) (clone EA-53; Sigma; St Louis, MO) and against FSTL1 (1/2000) (Abcam; Cambridge, UK). Immunoblots were developed with antimouse or antigoat IgG HRP, subjected to enhanced chemiluminescence detection reagents (Thermo Scientific; Rockford, IL), and exposed to film for appropriate times. Densitometry signals on radiographs were evaluated with Chemi Genius2 Bio Imaging System with Gene Tools software (Syngene; Frederick, MD).

**Statistical Analysis**

Summary statistics are presented as mean±SD for continuous variables and as number (percentage) for categorical variables. To address hypothesis 1, Student t test was used to compare serum FSTL1 levels between patients with chronic systolic HF and controls. These groups were matched a priori for age and sex. To address hypothesis 2, 1-way ANOVA was used to compare FSTL1 protein expression among 3 explanted human myocardial tissue groups: dilated cardiomyopathy, ischemic cardiomyopathy, and NF donor hearts. To assess hypothesis 3, multivariable regression models were used to study the association between FSTL1 and clinical markers while adjusting for confounders. To this end, we first carried out bivariate analyses in which FSTL1 levels were divided into 3 risk groups based on the tertiles of the observed FSTL1 distribution, where group 1 included patients with FSTL1 <14.3 arbitrary units, group 2 included patients with FSTL1 between 14.3 and 20.5 arbitrary units, and group 3 included patients with FSTL1 ≥20.5 arbitrary units. Demographics, clinical characteristics, and echocardiographic parameters were compared among the groups using ANOVA or χ² test as appropriate. We specified a priori that a factor must show a potential association (ie, P<0.10) with FSTL1 by bivariate analysis to be tested in a multivariable regression model. We then used a backward selection procedure with α=0.2 to keep variables in the model. In the multivariable regression models, FSTL1 was operationalized as a continuous variable. Linear multivariable regression was used for the LV mass index outcomes, whereas Cox multivariable regression was used for the time to death. Patients who survived through the end of the study period were considered censored observations. The assumption of proportional hazards was assessed before attempting Cox modeling. P≤0.05 was considered statistically significant. All reported P values are 2 tailed, and all CIs are computed at the 95% level. All analyses were performed using SAS version 9.2 (SAS Institute Inc; Cary, NC) statistical software.

**Results**

**FSTL1 and Clinical Characteristics**

Eighty-six patients with chronic systolic HF completed the study. Sixty-four percent of the patients were black, 60% were men, and 80% had hypertension. Mean LV ejection fraction was 21±9%, and mean LV mass and LV mass index were 284±89 g and 144±39 g/m², respectively, by echocardiography, thus demonstrating the presence of LV

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hypertrophy (LVH). Sixty-two percent of the controls were black and were age- and sex-matched (58±8 years and 57% men, respectively). Controls had no known cardiovascular disease, had normal blood pressure, and were not taking cardiovascular medications. Echocardiography demonstrated normal cardiac size and LV function. Mean LV ejection fraction was 63±5%, with mean LV mass and LV mass index of 169±36 g and 89±18 g/m², respectively.

Patients were on evidence-based therapy for systolic HF and the mean New York Heart Association functional class was 2.4±0.8 (Table 1). The chronic nature of these patients’ HF condition was demonstrated by the duration of HF symptoms at the time of enrollment (49±52 months; range, 1 to 237 months). The majority of patients were overweight/obese with a mean body mass index >30 kg/m². Mean creatinine level was 1.6±1.9 mg/dL with a Modification of Diet in Renal Disease glomerular filtration rate of 63.9±30 mL/min per 1.73 m². Serum FSTL1 levels were assessed by Western immunoblots and expressed in arbitrary units. Serum FSTL1 levels also were measured in age-matched healthy controls as previously described. Mean FSTL1 levels were increased in patients with HF compared with age- and sex-matched controls (19.8±7.2 versus 12.7±5 arbitrary units, P<0.01).

FSTL1 and Clinical and Echocardiography Variables
In the patients with chronic systolic HF, FSTL1 and BNP levels were significantly associated by bivariate analysis (P<0.001) (Figure 1), with the highest tertile of FSTL1 levels seen with the highest mean BNP levels (Table 2). FSTL1 levels were inversely associated with the presence of diabetes mellitus (P=0.04) across the tertiles. In addition, FSTL1 was significantly associated with left atrial size, posterior wall thickness, LV end-diastolic diameter, LV end-systolic diameter, LV mass, and LV mass index (Table 2).

FSTL1 and LV Mass
Change in LV mass is an indicator of cardiac remodeling. LVH was determined by LV mass indexed to body surface area. In patients with chronic systolic HF, both LV mass and LV mass index were significantly increased across the 3 tertiles of FSTL1 levels, with the greatest LV mass seen in the highest tertile of FSTL1 (P<0.001) (Figure 1, Table 2). By multivariate linear regression analysis, FSTL1 level was significantly associated with LVH (LV mass index) (slope, 6.7; 95% CI, 4.8 to 8.5) after adjusting for age, diabetes mellitus, BNP, left atrial size, and LV end-diastolic diameter (P<0.001). In other words, for each unit increase in FSTL1, our model predicted an increase of 6.7 g/m² in mean LV mass index.

FSTL1 and Mortality
Of the 86 patients with chronic systolic HF entered in this study between 2001 and 2004, 51% died by 2010; therefore, the relationship between FSTL1 levels and survival was investigated. In bivariate analysis, we found a marginally significant association between survival and FSTL1 levels (P=0.09). This significance disappeared when adjusting for confounders in multivariable Cox regression (P=0.26). By multivariable Cox regression analysis, after adjusting for left atrial size and LV end-diastolic diameter, we found that for each unit increase in FSTL1, the hazard of death increased by 2.8% (95% CI, 2.0% to 7.7%).

Measurement of FSTL1 Expression From Explanted Failing and NF LV
We measured FSTL1 protein expression in failing and NF heart samples. As shown in Table 3, subjects in the failing
group were older than those in the NF group. There were more women in the NF group. LV ejection fraction was <40% in the failing group. In the failing group, patients had either dilated cardiomyopathy or ischemic cardiomyopathy and received standard HF therapy that included diuretics and angiotensin-converting enzyme inhibitors or angiotensin receptor blockers. Some were taking digoxin, nitrates, dihydro-pyridine calcium channel blockers, and antiarrhythmics. None received β-blockers. Several patients in both groups received intravenous sympathomimetics such as dopamine or dobutamine and vasopressin.

Immunoblots demonstrated significantly increased signal intensity in the failing hearts (≈2.5-fold) compared to the NF hearts (NF, 1.0±0.2; dilated cardiomyopathy, 2.4±0.5; ischemic cardiomyopathy, 2.7±0.5; P<0.05 versus nonfailing) (Figure 2B, bottom). FSTL1 protein amount was normalized to α-actinin protein expression, which did not differ between groups (Figure 2B, top), and Ponceau S staining of blots confirmed equal protein loading (Figure 2A).

**Discussion**

The present study demonstrated the following: (1) Serum FSTL1 levels are significantly elevated in patients with chronic LV systolic HF versus age- and sex-matched healthy controls; (2) myocardial FSTL1 protein expression is significantly increased in the LV of explanted failing human hearts; (3) serum FSTL1 levels are predictive of LVH (as measured by LV mass index) in chronic human HF by multivariate linear regression analysis after adjusting for age, diabetes mellitus, BNP, left atrial size, and LV end-diastolic diameter; and (4) patients with chronic systolic HF with the highest FSTL1 levels had a higher risk of death. After adjusting for significant covariates, patients with the highest serum FSTL1 levels still had a trend for a worse prognosis when compared with those in the other groups.

In the present study, serum FSTL1 level was strongly associated with LVH, a measure of LV remodeling. Increased LV mass is an independent predictor of incident HF unrelated to prevalent or incident myocardial infarction. The present cohort comprised patients with chronic stable HF, where up to 80% had hypertension as a coexisting illness and 29% had hypertension as the primary etiology of their cardiomyopathy. Therefore, it is not surprising that LV mass was increased. BNP is a measure of LV wall stress and in our study, mean BNP levels were markedly elevated in patients with the highest FSTL1 levels. N-terminal proBNP levels predict mortality in patients with LVH. Thus, the finding in our study that FSTL1 predicts LVH is intriguing. Consequently, what is the significance of the relationship of serum FSTL1 to increased LV mass? LVH (increased LV mass) is a hallmark of hypertensive heart disease and is an important prognostic indicator of adverse cardiovascular outcomes. Additionally, it is an important target for monitoring hypertension therapy.

It is unknown whether the serum FSTL1 levels in these patients with chronic systolic HF simply reflect the underlying disease process or whether FSTL1 plays a maladaptive or compensatory role in modulating the pathogenesis of LV dysfunction. FSTL1 may play a mechanistic role in cardiovascular diseases because we previously demonstrated in experimental studies that it functions to protect against ischemia-reperfusion injury. We also have shown that FSTL1 is upregulated in ischemic skeletal muscle and that its overexpression promotes revascularization in a murine model of peripheral arterial disease. FSTL1 also promotes endothelial cell migration and suppresses apoptosis in vitro, indicating that it may function in a paracrine manner to promote endothelial cell function. Consistent with its beneficial effects, it has been reported that FSTL1 mRNA levels were increased in the failing human heart at the time of LVAD implantation and subsequently declined when LV function improved. Furthermore, elevated FSTL1 mRNA levels correlated with subsequent improved cardiac function after LVAD removal, suggesting an adaptive function of FSTL1.

It is of interest to compare the profile of FSTL1 with that of tumor necrosis factor (TNF-α), a mediator of inflammation. Elevated serum TNF-α level is a harbinger of poor prognosis in HF. Myocardial TNF-α expression is increased...
The role of FSTL1 as a mediator of inflammation has been suggested by its association with extracellular matrix-related and calcium-binding proteins. Increased FSTL1 level reduces matrix metalloprotease 1 and 3 expression in vitro, which may degrade extracellular matrix proteins. Similarly, FSTL1 overexpression inhibits proinflammatory cytokine expression and improves allograft survival. Thus, it is conceivable that FSTL1 level either reflects the inflammatory state of the heart or functions as a modulator of myocardial inflammation.

**Limitations**

Explanted myocardial tissue samples were not matched by age and sex because low numbers in general are inherent to explanted human heart tissue studies. Failing human heart tissues usually are obtained from an older population with disease. Conversely, controls with NF hearts are younger (who usually died of trauma or motor vehicle accidents) and healthy enough to be considered as a donor. Similarly,
samples could not be matched by sex, but when the 2 female NF controls were removed from the analysis, FSTL1 protein expression still remained significantly greater than that in NF donor hearts \((P<0.05)\). Despite these limitations, similarly to others, the data show that FSTL1 protein expression is increased in failing human myocardium.

Conclusions

Chronic systolic HF is associated with elevated circulating FSTL1 levels. FSTL1 is significantly associated with LVH, and its myocardial protein abundance is increased in the failing LV. Future studies are needed to determine its utility as a biomarker in chronic systolic HF and LV remodeling. In patients with acute coronary syndrome, elevated FSTL1 level was associated with poor outcome. The risk of death was higher in patients with acute coronary syndrome presenting with FSTL1 levels above the median than in patients with FSTL1 levels below the median. Similarly, the present data suggest that those patients with the highest FSTL1 levels have decreased survival. The study is limited by the sample size, which is relatively small, and larger prospective studies will be required to better define the role of serum FSTL1 as a prognostic biomarker for HF.

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Disclosures

None.

References

CLINICAL PERSPECTIVE

Increasing evidence shows that the heart secretes factors to maintain its performance and coordinate cellular activities in response to stress or injury. The identification and study of these cardiac-secreted factors is significant because they may provide information about intertissue communication within or outside the heart. In addition, these secreted factors may serve as useful therapeutic or diagnostic tools. Follistatin-like 1 (FSTL1) is a secreted glycoprotein that is markedly upregulated by cardiac stress in experimental models of ischemia-reperfusion injury and myocardial infarction. Recently, FSTL1 levels were found to be increased in humans with acute coronary syndrome and associated with all-cause mortality. Similarly, cardiac FSTL1 gene expression was increased in patients with end-stage heart failure, which subsequently declined when left ventricular function improved. Thus, these findings suggest that FSTL1 can serve as a cardiac tissue-derived marker of cardiovascular disease. We found that FSTL1 levels can be detected in human serum, are elevated in patients with left ventricular systolic heart failure, and are associated with left ventricular hypertrophy. The highest levels of FSTL1 are associated with decreased survival. The role of FSTL1 in heart failure is unknown. Future larger studies on the role of FSTL1 as a biomarker in chronic systolic heart failure are needed.
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