Associations between Endothelin-1 and Adiponectin in Chronic Heart Failure

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Endothelin-1 · Adiponectin · Heart failure · Cardiomyocyte · Hypertrophy · NT-proBNP

Abstract

Objectives: Endothelin-1 (ET-1) induces cardiac hypertrophy, whereas adiponectin may elicit protective effects in the vasculature and myocardium. We therefore evaluated the relationship between plasma ET-1 and adiponectin levels in heart failure (HF) patients, and the association between adiponectin expression and ET-1-induced hypertrophy of human cardiomyocytes (HCM) in vitro. Methods: One hundred seventeen patients with chronic HF were enrolled into this study. A group of 7 patients with end-stage HF undergoing heart transplantation was included in the histopathological study. Baseline clinical evaluations and laboratory measurements were performed. HCM cultures were studied to investigate the effect of ET-1 on cell size and adiponectin expression. Results: Plasma ET-1, adiponectin, and N-terminal pro-B-type natriuretic peptide (NT-proBNP) increased with the severity of HF. Higher New York Heart Association functional class, plasma ET-1, adiponectin, and NT-proBNP levels were significant predictors of adverse outcomes in these patients. The myocardial expression of adiponectin was significantly higher in the recipient hearts of patients undergoing emergency or urgent heart transplantation. In cell culture, ET-1 significantly increased cell size and adiponectin expression in HCM. Conclusions: Adiponectin was significantly elevated in HF and was significantly associated with ET-1. The study provides a basis for further investigation of ET-1 and adiponectin modulation as a therapeutic strategy for ventricular remodeling in HF.

Introduction

Adiponectin is one of the adipokines that play a role in the modulation of glucose metabolism, insulin resistance, and inflammation [1, 2]. Recognized in 1995 as a novel protein produced mainly by adipocytes [3], adipon-
Adiponectin has been observed to circulate in increased concentrations in subjects with chronic heart failure (HF) [4]. Moreover, high adiponectin levels are a predictor of mortality in patients with HF [4, 5].

Several reports have suggested possible roles for endothelin-1 (ET-1) [6] in the regulation of adiponectin production [6–8]. The role of ET-1 is still unclear because both stimulation [6] and suppression [7, 8] of adiponectin have been reported in adipocytes. Recent studies suggest that adiponectin is not only an adipocyte-specific endocrine molecule with cardioprotective effects but it is also expressed in cardiomyocytes [9–12]. However, the biological significance of this locally produced adiponectin remains unclear. These effects could result from a change in adiponectin production or adiponectin clearance. Furthermore, it is also unclear which mechanism(s) result in the observed upregulation in circulating adiponectin levels of patients with advanced HF.

ET-1, a potent vasoconstrictor peptide produced by the vascular endothelium, is also synthesized and secreted by cardiomyocytes [13–16] and induces hypertrophy of cardiomyocytes [16–18]. The production of ET-1 is increased both in the hypertrophied heart and in the failing heart [19, 20], which suggests that ET-1 plays an important role in the development of cardiac hypertrophy and HF. Moreover, adiponectin has cardioprotective actions, and adiponectin receptors AdipoR1 and AdipoR2 mediate the suppressive effects of adiponectin on ET-1-induced hypertrophy in cultured cardiomyocytes [21].

Therefore, we hypothesized that a relationship between plasma levels of ET-1 and adiponectin exists, and their measurements may provide prognostic information in patients with chronic HF. Furthermore, we hypothesized that in myocardium, adiponectin may act as a cardiac endothelial inhibitor for ET-1, and the association between adiponectin expression and ET-1-induced hypertrophy of HCM were studied in vitro.

**Patients and Methods**

**Study Population**

From January 2005 to December 2006, 117 consecutive patients with proven systolic HF, who had been referred to the Division of Cardiology for clinical HF management and/or transplantation evaluation, were recruited. All of them had New York Heart Association (NYHA) functional classification II to ambulatory class IV despite optimal medical treatment, and those who had a left ventricular ejection fraction (LVEF) <40% by echocardiography or left ventriculography with radionuclide or contrast medium within 7 days were also eligible for enrollment. Informed consent was obtained from the patients who participated in this study. The study was approved by the Ethical Review Board for Research [CHGH-IRB No. (165) 98-21] at Cheng-Hsin General Hospital, Taipei, Taiwan. The investigation conformed to the principles outlined in the Declaration of Helsinki for use of human tissues or subjects [22].

Patients were excluded if they had hemodynamically significant obstructive valvular disease, cor pulmonale, myocarditis, constrictive pericarditis, or congenital heart disease, or if they had experienced myocardial infarction or unstable angina within the 6 months leading up to enrollment or were scheduled to undergo major surgery prior to study enrollment. Those who fitted into any of the following categories, up to 1 week before study entry, were not allowed to enroll either: deviation in NYHA functional class, administration of intravenous inotropes or change in antifailure medications. In addition, any patients who had been resuscitated from sudden death, experienced severe comorbidities, such as significant hepatic or renal diseases, uncontrolled thyroid disease, or drug or alcohol abuse, or had infection or an inflammatory illness, such as sepsis, malignancy, arthritis, or connective tissue disease, were excluded.

A group of 7 patients with end-stage HF undergoing heart transplantation were included in the histopathological study. Clinically, they were classified as emergency transplantation candidates (hospital-bound recipients requiring intravenous inotropes and/or mechanical support of the circulation) or elective transplantation candidates (ambulatory outpatients).

**Baseline Clinical Evaluation**

Baseline clinical evaluations were performed by a physician on study patients with HF. Information gathered included medical history, e.g. medications used, physical examination, NYHA classification based on patient information, estimated glomerular filtration rate determined by the Modification of Diet in Renal Diseases formula, echocardiography, and measurements of pulmonary capillary wedge pressure and cardiac index by right heart catheterization.

**Blood Sampling and Laboratory Measurements**

After a minimum of 8 h of overnight fast and 20 min of supine rest, venous blood was drawn during right heart catheterization (central vein) or collected from an indwelling catheter into vacuum EDTA tubes (peripheral vein) at bedside. All samples were processed immediately. The plasma were separated by centrifugation and then frozen to −20°C and stored at that temperature until further analysis. The time intervals between blood sampling and LVEF studies were less than 1 week in all cases.

Assays for ET-1, adiponectin, and N-terminal pro-B-type natriuretic peptide (NT-proBNP), another well-established powerful risk marker in HF, were done concurrently to minimize the possible inaccuracy due to repeated freeze-thaw cycles. The NT-proBNP levels were determined with Roche Elecsys® NT-proBNP (Roche Diagnostics), a quantitative electrochemiluminescence immunoassay. Plasma concentrations of ET-1 and adiponectin were measured with commercial sandwich enzyme-linked immunosorbent assays (R&D, Minneapolis, Minn., USA). The intra- and interassay coefficients of variation for each factor in our laboratory were around 5 and 10%, respectively.
Clinical Follow-Up

All patients were followed either in-hospital or through regular outpatient visits. Clinical information regarding major adverse cardiac events (cardiac death, requirement for heart transplantation, or hospitalization with a primary diagnosis of worsening HF) during a median follow-up period of 206 days was provided by the cardiologists in charge, without knowledge of biomarker levels.

Immunohistochemistry and Western Blot Analysis

For immunohistochemistry on the formalin-fixed paraffin-embedded samples of myocardial tissues obtained from recipient hearts, 5-μm sections were blocked by 5% serum at room temperature for 1 h. Primary mouse anti-adiponectin antibody (1:100; Chemicon, Temecula, Calif., USA) was added onto the sections and incubated at 4°C overnight. After washing in PBS, the sections were incubated with goat anti-mouse secondary antibody (1:400; Sigma, St. Louis, Mo., USA) at room temperature for another hour and later, after PBS washing, 50 μg/ml propidium iodide (Sigma) was added and then incubated for 1 min at room temperature. Following another wash with PBS, the sections were mounted using Vectashield mounting medium (Vector) and examined with a Leica TSC SP2 confocal laser scanning microscope.

Western blot analyses were performed as described previously [23, 24]. Briefly, the 25- to 40-μg protein subjected to 12% SDS-polyacrylamide gel electrophoresis was transferred onto PVDF membranes and then underwent blotting. Having been blocked with 5% skim milk in Tween-20/PBS, blots were incubated with various primary antibodies. Blots were later incubated with horseradish peroxidase-conjugated secondary antibodies. Its signal was detected using Chemiluminescence Reagent Plus (NEN, Boston, Mass., USA). The intensity of each band was quantified using a densitometer.

Culture of Human Cardiomyocytes

Human cardiomyocytes (HCM; catalog No. 6200) were purchased from ScienCell Research Laboratories (Carlsbad, Calif., USA) and grown in Cardiac Myocyte Medium (CMM; catalog No. 6201) supplemented with 5% fetal bovine serum, 1% cardiac myocyte growth supplement (CMGS; catalog No. 6252), and 1% penicillin/streptomycin solution (P/S; catalog No. 0503). Cells were grown in poly-L-lysine-coated culture dishes in a humidified incubator with 5% CO₂ at 37°C. The culture medium was renewed every 3–4 days. In all experiments, cell passage was between 3 and 8. HCM were approved for in vitro experimental use by the Safety Committee for Biological Experiments at Kaohsiung Medical University.

Measurement of the Cardiomyocyte Surface Area

HCM were viewed using a video camera (Nikon) attached to a microscope and projected onto a monitor. The surface area was determined using image analysis software (MetaMorph Imaging System, Meta Imaging Series 4.5) and calculated as the mean of 100–120 cells from randomly selected fields.

Statistical Analyses

All values were expressed as means ± SEM. The biomarker levels in these patients did not follow a normal distribution and were therefore expressed as medians (25th to 75th percentiles).

The patients were divided into 3 groups based on their NYHA functional classes. Comparisons of clinical and hemodynamic data among these 3 groups were performed by means of a 1-way analysis of variance (ANOVA) test. Comparisons of the levels of biomarkers among these 3 groups were performed by means of a Kruskal-Wallis test. Linear regression analysis was used to determine the correlation between levels of ET-1, adiponectin, NT-proBNP, and the body mass index (BMI). Kaplan-Meier analyses of cumulative event-free rates in HF patients, stratified into 3 groups on the basis of their NYHA functional classes or tertiles of plasma levels of biomarkers, were compared. The differences between event-free curves were evaluated using a log-rank test. In a laboratory study, comparisons between multiple groups were determined by means of a 1-way ANOVA followed by Dunnett's test. p < 0.05 was considered statistically significant.

Results

Clinical Characteristics and Circulating Levels of ET-1, Adiponectin, and NT-proBNP in HF Patients

The baseline characteristics of the 117 patients with HF, divided into 3 groups based on their NYHA functional classes, are shown in table 1. Upon entry, 35 patients were in NYHA class II, 43 in class III, and 39 in ambulatory class IV. No significant differences in age, sex, BMI, etiology of HF, or estimated glomerular filtration rate among the 3 groups were detected; however, there were more men than women in this sample.

The mean LVEF and the hemodynamic parameters were significantly poorer, and the concentrations of ET-1, adiponectin, and NT-proBNP increased significantly with the severity of HF; especially, in the NYHA functional class IV patients.

Correlations between ET-1, Adiponectin, NT-proBNP, and BMI

Adiponectin and NT-proBNP were significantly and positively correlated (fig. 1a; p < 0.0005, ρ = 0.340) and they were negatively correlated with BMI (fig. 1b, c; p < 0.001 and p < 0.005, ρ = –0.316 and –0.281, respectively). A positive correlation was revealed with the concentrations of ET-1, estimated with both adiponectin (fig. 1d; p < 0.05, ρ = 0.241) and NT-proBNP (fig. 1e; p < 0.0005, ρ = 0.333). However, no significant association was found between ET-1 serum concentrations and BMI (fig. 1f; p = 0.118, ρ = –0.151).

Prognosis for Freedom from HF Hospitalization

The median follow-up period was 206 days (128–355 days, 25th to 75th percentiles). There was a 42% (49/117) overall event rate in the HF population. Nine of the 117
patients died of cardiac causes (3 died of sudden death without premonition of any progression symptoms and presumably due to arrhythmia, and the other 6 died of intractable end-stage HF) during the follow-up period. Ten patients underwent heart transplantation, and 30 of them were readmitted for worsening HF.

Kaplan-Meier analyses of cumulative event-free rates in HF patients (free from HF hospitalization), stratified into 3 groups on the basis of their NYHA functional classes or tertiles of plasma levels of biomarkers, are shown in figure 2. Patients with a higher functional class (fig. 2a) and higher plasma levels of ET-1 (fig. 2b), adiponectin (fig. 2c), and NT-proBNP (fig. 2d) were significantly associated with higher adverse event rates (HF hospitalization) during follow-up (all p < 0.05).

Cardiac Adiponectin Protein Expression in Recipient Hearts

Among the 10 end-stage HF patients who underwent heart transplantation, 7 patients gave us their consent for research with cardiac samples. Expression of adiponectin in the myocardium was assessed by Western blot and immunohistochemistry analyses. In comparison with those who underwent elective heart transplantation (n = 2), the expression levels of adiponectin protein in myocardium from the recipient hearts of patients who underwent emergency or urgent transplantation (n = 5) increased significantly according to Western blot analysis (fig. 3a). Immunohistochemical staining also revealed similar findings. Representative photographs of myocardium tissue, stained with anti-adiponectin antibodies, are displayed in figure 3b.

ET-1 Increases Cell Size and Adiponectin Expression in Cardiomyocytes in vitro

To examine the effects of ET-1 in cardiomyocytes at the cellular level, we used in vitro cell culture to study cardiomyocyte hypertrophy and adiponectin expression with HCM. ET-1 stimulation for 48 h caused a significant increase in cardiomyocyte size (fig. 4a). In the meantime, incubation of HCM with ET-1 significantly increased the protein expression of adiponectin in a time- and dose-dependent manner (fig. 4b).

Discussion

Several new observations were made in our study. The circulating levels of ET-1, adiponectin, and NT-proBNP significantly increased with the severity of HF; evidently, these were significant predictors of adverse clinical outcomes. ET-1, adiponectin, and NT-proBNP were also positively correlated with each other. However, only the levels of adiponectin and NT-proBNP were negatively cor-
Fig. 1. Scatter-plots of the association between log-transformed values of plasma NT-proBNP and plasma adiponectin levels (a), the association between BMI and log-transformed values of plasma adiponectin levels (b), the association between BMI and log-transformed values of plasma NT-proBNP levels (c), the association between log-transformed values of plasma adiponectin and plasma ET-1 levels (d), the association between log-transformed values of plasma NT-proBNP and plasma ET-1 levels (e), and the association between BMI and plasma ET-1 levels (f).
related with BMI. In the cardiac tissues obtained from patients who underwent heart transplantation, adiponectin was markedly upregulated in the left ventricle with severe HF. In the cultured HCM, ET-1 caused a significant increase in myocyte size and adiponectin protein expression. Altogether, these results suggest that high ET-1 may be a prognostic predictor of poor clinical outcomes in HF patients, and part of this relation may be mediated by adiponectin and NT-proBNP levels. Furthermore, the in vitro study suggests that adiponectin expression in myocardium probably plays an important role in ET-1-induced cardiomyocyte hypertrophy.

Previous studies have demonstrated elevated plasma concentrations of ET-1 in patients with various levels of severity and etiology of chronic HF [25, 26]. The mechanism which increases the ET-1 plasma concentration may be the increased peptide secretion and release of endothelium from damaged cells within the cardiac cavities [15]. The increased ET-1 plasma concentration in patients with HF may lead to cardiac hypertrophy and fibrosis [16, 19–21]. ET-1 may also trigger the inflammation process through stimulation of cytokine production [16] and may influence the electrical remodeling of cardiomyocytes through an increased intracellular calcium ion concentration [26]. Moreover, the in-
fluence of ET-1 on the renin-angiotensin-aldosterone system also plays an important role in the structural, electrical, and neurohormonal remodeling of myocardium [27]. Our finding that the circulating levels of ET-1, adiponectin, and NT-proBNP significantly increased with the severity of HF and were significant predictors of adverse clinical outcomes is in accordance with previous studies [4, 25]. However, to the best of our knowledge, this study is the first to examine the associations between ET-1 and adiponectin, NT-proBNP, and BMI simultaneously.

The results of immunohistochemistry and Western blot analysis showed that the expression levels of adiponectin protein significantly increased in the myocardium from recipient hearts. This is interesting because the observed upregulation of adiponectin protein in cardiac tissue could be secondary to the increased serum concentrations of ET-1, a possible regulator of adiponectin expression [6]. Since adiponectin is present in damaged myocytes and adiponectin is synthesized and secreted by isolated HCM [9–12], the findings suggest the existence of a local cardiac adiponectin autocrine system which is likely regulated by systemic cytokines including ET-1 serum levels.

It is well known that adiponectin exhibits protective properties in the heart and blood vessels [28–33]. As for the heart, adiponectin serves as a regulator of cardiac injury through modulation of anti-inflammatory and pro-survival reactions and functions and to inhibit hypertrophic remodeling [32]. Future studies will be required to identify the oligomeric isoform(s) of adiponectin which confer cardioprotection and clarify the receptor-mediated signaling mechanisms inhibiting myocardial apoptosis, inflammation, and hypertrophy. Evaluation of the molecular and cellular mechanisms of adiponectin action may lead to a better understanding of how adiponectin affects the heart and permits the development of novel approaches to treat heart disease.

In the in vitro study, we further demonstrated ET-1-induced adiponectin expression in cardiomyocytes; future work will be needed to gain additional insight into the mechanism of the ET-1-mediated regulation of adiponectin expression. As mentioned earlier, the finding of adiponectin expression in cardiomyocytes is likely counterintuitive; it has been found that pressure overload in adiponectin-deficient mice results in enhanced concentric cardiac hypertrophy and adiponectin-inhibited agonist-stimulated hypertrophy in cultures of cardiac myo-
Since adiponectin exhibits antihypertrophic effects on cardiomyocytes \cite{21, 28, 29}, our data suggest a compensatory mechanism of cardiomyocytes in response to ET-1 stimulation.

Other limitations of the present study should be mentioned. In addition to ET-1, adiponectin, and NT-proBNP, other important adipocytokines, such as tumor necrosis factor-\(\alpha\), may have a stronger association with prognosis in patients with HF. We did not measure changes in weight or ET-1, adiponectin, or NT-proBNP during follow-up and, hence, no causality of the interrelationship between these parameters can be determined.

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**Fig. 4.** ET-1 increases cell size and adiponectin expression in cultured HCM. ET-1 stimulation for 48 h caused a significant increase in cardiomyocyte size (a). Incubation of HCM with ET-1 significantly increased the protein expression of adiponectin in a time- and dose-dependent manner (b). Three independent experiments yielded similar results. The summarized data (mean ± SEM) from 3 separate experiments are shown in the bar graph. *p < 0.05 compared to the control group.
from the present study. The relatively small number of deaths requires that caution be exercised in the interpretation of the results, especially while pondering the complex interaction between ET-1, adiponectin, NT-proBNP, and BMI. Finally, because this is the pilot study for examining ET-1 and adiponectin in relation to prognosis in HF, its findings can only be confirmed in future studies, and the precise regulatory mechanisms for the myocardial expression of adiponectin in cardiomyocyte hypertrophy need to be further examined.

Conclusion

The present study shows, for the first time, that though a high ET-1 level can be a predictor of adverse clinical outcomes in HF patients, this relation may partially be mediated by adiponectin and NT-proBNP levels. An important finding of the in vitro study proves that adiponectin expression plays an important role in ET-1-induced cardiomyocyte hypertrophy. Therefore, our research identified ET-1 as a candidate for targeting clinical HF and provides a basis for further investigation of adiponectin modulation as a therapeutic strategy for cellular hypertrophy in cardiomyocytes in HF.

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Conflict of Interest

None declared.

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