Plasma Angiopoietin-1, Angiopoietin-2, and Angiopoietin Receptor Tie-2 Levels in Congestive Heart Failure

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OBJECTIVES	The goal of this research was to test the hypothesis that plasma angiopoietin (Ang-1), its soluble receptor tie-2, and Ang-2 levels would be abnormal in patients with acute and chronic congestive heart failure (CHF) when compared with healthy controls.
BACKGROUND	Increased plasma vascular endothelial growth factor (VEGF) in CHF is suggestive of excess angiogenesis—possibly driven by tissue hypoxia. However, other growth factors also have a major role in angiogenesis, such as those of the angiopoietin family (e.g., Ang-1, which exerts its activity via its receptor, tie-2, and Ang-2).
METHODS	We recruited 39 patients with acute CHF (mean age 67 ± 10 years), 40 patients with chronic CHF (mean age 63 ± 9 years), and 17 healthy controls (mean age 67 ± 7 years), all in sinus rhythm. Citrated plasma was analyzed for Ang-1, Ang-2, tie-2, and VEGF by enzyme-linked immunosorbent assay.
RESULTS	Angiopoietin-2 ($p < 0.001$), tie-2 ($p < 0.05$), and VEGF ($p < 0.05$) levels were all higher in acute CHF compared with controls. The Ang-2 levels were higher in acute CHF compared with chronic CHF ($p < 0.001$), but there were no significant differences in Ang-1 levels between the groups. The principal significant correlations were between Ang-2 and tie-2 (Spearman, $r = 0.407$; $p < 0.0001$) and between Ang-2 and ejection fraction ($r = -0.241$, $p = 0.043$). Although only marginally raised, levels of VEGF correlated with both Ang-2 ($r = 0.468$, $p < 0.001$) and tie-2 ($r = 0.569$, $p < 0.001$).
CONCLUSIONS	We have demonstrated abnormal levels of Ang-2 and tie-2, but normal Ang-1, in both CHF patients. These abnormalities may, alongside VEGF, indicate a role for these angiogenic factors in the pathophysiology of CHF. (J Am Coll Cardiol 2004;43:423–8) © 2004 by the American College of Cardiology Foundation

Congestive heart failure (CHF) is characterized by poor cardiac output and, consequently, tissue ischemia, the latter being a powerful stimulus for angiogenesis. Our recent demonstration of raised plasma levels of the angiogenic vascular endothelial growth factor (VEGF) in CHF supports the concept that ongoing angiogenesis is probably present in this condition (1). More recently, oncology research has identified other growth factors, such as those of the angiopoietin (Ang) family, that appear to have a major role in angiogenesis and metastasis (2–4). The roles of two members of this family, Ang-1 and Ang-2, are becoming understood and may act alongside VEGF. The latter is responsible for the initiation of angiogenesis through recruitment and proliferation of endothelial cells, a process that Ang-1 potentiates (5).

Angiopoietin-1 and -2 play modulatory roles by binding a common receptor, the endothelial cell-specific tyrosine kinase receptor tie-2. Unlike VEGF, Ang-1 and Ang-2 do not have mitogenic/proliferative properties. More specifi-

cally, Ang-1 is a chemotaxin (6) and, in conjunction with VEGF, recruits endothelial cells to initiate and accelerate angiogenesis. In a murine mammary carcinoma model, Ang-1 is secreted and then bound to the extracellular matrix (7) where it is ideally situated to mediate cell-to-cell and cell-to-matrix interaction of capillaries (8,9).

In the presence of VEGF, Ang-2 promotes a rapid increase in capillary diameter, remodeling of the basal lamina and new vessel growth evidenced by sprouting of existing blood vessels, a process that can also be stimulated by Ang-1 (10). In contrast, if VEGF is inhibited, Ang-2 leads to endothelial cell death and vessel regression (11). Therefore, Ang-1 potentiates VEGF in the early process of angiogenesis (i.e., endothelial cell recruitment and proliferation), as well as adhesion to the basal lamina (10), whereas Ang-2 promotes vascular remodelling (12). Although hypoxia is known to be a powerful stimulant for angiogenesis, several studies have shown that hypoxia itself does not stimulate the expression of Ang-1 (13,14), which is also known to have antiapoptotic properties via the PI-3 kinase/Akt/survivin pathway (15).

As hypoxia, endothelial dysfunction/damage (16) and raised VEGF (1) are present in CHF, we hypothesized alterations in the angiopoietin family, specifically, Ang-2. However, as hypoxia does not stimulate the expression of Ang-1 (13,14), we also hypothesized that Ang-1 would not

Abbreviations and Acronyms

Ang	= angiopoietin
CHF	= congestive heart failure
CV	= coefficient of variation
ELISA	= enzyme-linked immunosorbent assay
HMG-CoA	= hydroxymethylglutaryl-coenzyme A
LVEF	= left ventricular ejection fraction
NYHA	= New York Heart Association
RNA	= ribonucleic acid
VEGF	= vascular endothelial growth factor

be altered in CHF. This lack of stimulated Ang-1 release as an "endothelial survival factor" could be partly responsible for the profound endothelial abnormalities observed in CHF. We tested our hypothesis by developing an assay for quantification of Ang-1 and applying this assay (and other established assays for quantification of Ang-2, tie-2, and VEGF) in a simple cross-sectional study of acute and chronic CHF patients, compared with healthy matched controls.

METHODS

Patients and controls. We recruited 39 patients with acute CHF, 40 patients with chronic CHF, and 17 healthy controls, all of whom were in sinus rhythm. Congestive heart failure was defined according to European Society of Cardiology guidelines (17). All patients with acute CHF had evidence of pulmonary edema on chest X-ray in addition to clinical signs of heart failure. Subjects with CHF

were classified according to the New York Heart Association (NYHA) criteria with functional class I to II being no, or mild, symptoms and class III to IV being moderate to severe symptoms. Chronic CHF was defined as patients being in a stable NYHA functional class for at least three months and were recruited from outpatient clinics.

Echocardiography was undertaken either during admission or within three months from the time of admission, and only patients with a documented left ventricular ejection fraction (LVEF) of <40% (either using M-mode transthoracic echocardiography or, if there was significant regional wall motion abnormality, by Simpson's method) were included in our analysis.

Exclusion criteria were atrial fibrillation; concomitant acute coronary syndrome; infection or pyrexial illness; recent (<3 months) myocardial infarction or stroke; chronic and systemic illnesses including renal failure, hepatic impairment, cancer, and inflammatory connective tissue disease; inflammatory bowel disease; use of oral steroids; and hormone replacement therapy. Healthy control subjects were recruited from among healthy hospital staff; spouses of patients; and from subjects attending the hospital for hernia repairs, varicose vein procedures, or other relatively minor operations. All healthy control subjects had no clinical evidence of vascular, metabolic, neoplastic, diabetic, or inflammatory disease on careful history, examination, and routine laboratory tests. Clinical characteristics of patients and controls are summarized in Table 1.

Patients with acute CHF were venesected within 24 h of admission to hospital, whereas the separate cohort of

Table 1. Baseline Characteristics of the Study Population

	Acute CHF	Chronic CHF	Controls	p Value
n	39	40	17	–
Gender (M:F)	27:12	29:11	4:13	0.001
Age (yrs)	67 ± 10	63 ± 9	67 ± 7	0.254
"Usual" NYHA class				
I–II	5 (13%)	22 (55%)	—	< 0.0001
III–IV	34 (87%)	18 (45%)	—	< 0.0001
LVEF (%)	30 (27–35)	30 (30–35)	—	0.688
Comorbidity				
IHD	23 (59%)	33 (83%)	0	< 0.0001
HT	22 (56%)	14 (35%)	0	< 0.0001
DM	15 (38%)	15 (38%)	0	0.93
Cholesterol	4.2 ± 0.2	4.7 ± 0.2	5.7 ± 0.3	< 0.0001
Cigarette smoking (current)	3 (7.7%)	6 (15%)	1 (5.9%)	0.097
Previous CVA/TIA	0 (0%)	2 (5%)	0	0.239
Treatment				
ACE inhibitor or angiotensin receptor blocker	27 (69%)	38 (95%)	0	< 0.001
Beta-blocker	11 (28%)	20 (50%)	0	0.082
Anti-platelet agent	22 (56%)	30 (75%)	0	0.082
Warfarin	8 (21%)	5 (13%)	0	0.337
Statin	11 (28%)	25 (63%)	0	0.002

Data expressed as mean ± 1 SD or n (%) unless stated. p values based on chi-square test, except age and cholesterol by analysis of variance (acute CHF vs. chronic CHF vs. controls) and LVEF by Mann-Whitney *U* test (acute CHF vs. chronic CHF).

ACE = angiotensin-converting enzyme; CHF = congestive heart failure; CVA = cardiovascular accident; DM = diabetes mellitus; HT = hypertension; IHD = ischemic heart disease (prior myocardial infarction or symptoms of angina pectoris with positive exercise tolerance test or coronary disease on angiography); LVEF = left ventricular ejection fraction; NYHA I to IV = New York Heart Association classification (I, asymptomatic; IV, symptoms at rest or with minimal exertion); TIA = transient ischemic attack; — = not estimated.

patients with chronic CHF were venesected in the outpatient research clinic. Citrated plasma was obtained from venous blood by centrifugation at 3,000 rpm (1,000 g) for 20 min at 4°C. All aliquots were stored at −70°C to allow batch analysis.

Laboratory methods. Growth factors and receptors were measured by enzyme-linked immunosorbent assay (ELISA) as follows (1,18–20): mouse anti-human Ang-1, biotinylated goat anti-human Ang-1 and recombinant human Ang-for the Ang-1 assay, mouse anti-human Ang-2, biotinylated mouse anti-human Ang-2 and recombinant Ang-2 for the Ang-2 assay, goat anti-human tie-2, biotinylated goat anti-human tie-2 and recombinant human tie-2/Fc chimera for the tie-2 assay, and rabbit anti-human VEGF, biotinylated goat anti-human VEGF, biotinylated goat antihuman Flt-1 and recombinant human VEGF-165 for the VEGF assays (all obtained from R&D Systems, Abingdon, Oxfordshire, United Kingdom). The precise methods for VEGF, tie-2, and Ang-2 are available elsewhere (1,19,20).

Development of the Ang-1 assay. As the Ang-1 assay is novel (18), full details are provided. The ELISA system was based on a sandwich noncompetitive ELISA, consisting of an anti-human Ang-1 antibody (capture antibody) and a biotinylated goat anti-human Ang-1 (detection system). ExtrAvidin peroxidase (Sigma, Poole, United Kingdom) was used to amplify the antibody-antigen reaction, and the color was developed using ortho-phenylenediamine dihydrochloride tablets (Sigma). Mouse anti-human Ang-1 antibody was adsorbed onto 96-well microtitre immunoassay plates for a minimum of 15 h at 4°C (i.e., overnight) at concentrations ranging from 0.5 to 10 μg/ml. Biotinylated goat anti-human antibody at concentrations ranging from 250 to 500 ng/ml was combined to measure plasma Ang-1. Optimum titres were determined using checkerboard titration, seeking high optical density from standards and minimum optical density from blanks. Standard curves were generated using recombinant human Ang-1 (rHAng-1) protein at 0 to 1,000 ng/ml.

The final protocol for Ang-1 ELISA is as follows (18): First, 96-well microtitre immunoassay plates (Dynex Labsystems, Ashford, Middlesex, United Kingdom) were coated with 100 μl/well of 2 μg/ml mouse anti-human Ang-1 in 0.05 mol/l carbonate/bicarbonate buffer (pH 9.6) for a minimum of 15 h at 4°C. Plates were then washed three times in assay buffer (0.05% Tween 20 in 0.1 mol/l phosphate buffered saline, pH 7.2) and in between each subsequent incubation period. After blocking for 2 h at room temperature with 5% dried powdered milk (Marvel, Cadbury, Birmingham, United Kingdom) in phosphate buffered saline-Tween (200 μl/well), recombinant human Ang-1 standards (ranging from 0 to 100 ng/ml) and plasma samples were added in duplicate (100 μl/well) for 2 h at room temperature; 100 μl/well biotinylated detection antibody (500 ng/ml) was then added for a further 2 h at room temperature. ExtrAvidin peroxidase (1:1,000 dilution)

was added for 45 min at room temperature (100 μl/well) before the development of color with 10 mg orthophenylenediamine dihydrochloride dissolved in 20 ml 0.05 mol/l citrate buffer (pH 5.0) and 10 μl 1 mol/l hydrogen peroxide. The reaction was stopped using 1 mol/l hydrochloric acid and the absorbance read at 492 nm.

The intra-assay coefficient of variation was 3.87% at 20 ng/ml Ang-1 (n = 24), and 4.13% at 40 ng/ml. Interassay coefficient of variation was 9.45% at 20 ng/ml (n = 8) and 9.78% at 40 ng/ml (n = 7). The lower limit of sensitivity for the assay was a concentration of 1 ng/ml Ang-1.

Power calculation and analysis. We have previously reported plasma VEGF to be raised by a factor of 2 in 53 patients with chronic CHF and almost 3 in 77 with acute CHF, compared with 37 healthy controls, a difference that was most significant (p < 0.001) (1). Therefore, seeking more modest significance (analysis of variance, p < 0.05) in the present study, we set our power calculation at a minimum of 17 subjects per group (sample size = 51) to enable us to detect a difference of 1 SD with 1-β = 0.8 and p < 0.05 between groups. Data were analysed by the Shapiro-Wilks test to determine distribution. Normally distributed data are expressed as mean and SD. As the data for LVEF, VEGF, and Ang/tie-2 were not normally distributed, values are expressed as median (interquartile range). Baseline cross-sectional data between acute CHF patients, chronic CHF outpatients, and healthy controls were analyzed by analysis of variance, the Mann-Whitney or the Kruskal-Wallis test as appropriate, with between group comparisons by Tukey's post-hoc test (after log transformation). Correlations were performed using Spearman's rank correlation method. Stepwise multiple regression analysis was used to determine the predictors of abnormal research indexes. A value of p < 0.05 was considered statistically significant.

RESULTS

Cross-sectional analyses and correlations. Median plasma levels of Ang-1 in CHF patients were not significantly different than healthy controls (p = 0.07), but median Ang-2 levels were significantly elevated in CHF (p < 0.0001), with significant differences in Ang-2 levels between acute CHF, chronic CHF, and healthy controls (each, p < 0.05) (Table 2, Fig. 1). There was a significant overall variance in levels of tie-2 levels between the three groups, with the difference between the controls and the acute CHF patients being significant (p < 0.05) (Table 2, Fig. 2). There was also significant overall variance in levels of VEGF (p = 0.04), but there were no statistically significant intergroup differences.

The principal significant correlations were between Ang-2 and tie-2 (Spearman r = 0.407, p < 0.0001) and between Ang-2 and ejection fraction (r = −0.241, p =

Table 2. Ang-1, Ang-2, Ang Receptor Tie-2, and VEGF Levels in CHF

Variable	Acute CHF (n = 39)	Chronic CHF (n = 40)	Controls (n = 17)	p Value
Ang-1 (ng/ml)	10 (4–28)	15 (8–31)	8 (5–17)	0.07
Ang-2 (ng/ml)	16 (10–20)	7.6 (6–11)	5 (4.0–5.8)	< 0.0001
Tie-2 (ng/ml)	18 (14–26)	14 (12–24)	10 (8–13)	0.001
VEGF (pg/ml)	300 (200–600)	250 (40–575)	220 (80–270)	0.04

Data expressed as median (interquartile range); p value from the Kruskal-Wallis test.
Ang = angiopoietin; CHF = congestive heart failure; VEGF = vascular endothelial growth factor.

0.043). Although only marginally raised, levels of VEGF correlated with both Ang-2 ($r = 0.468$, $p < 0.001$) and tie-2 ($r = 0.569$, $p < 0.001$). There were no statistically significant differences in plasma levels of any of the research indexes when the subgroups of patients with NYHA functional class II were compared with NYHA functional class III/IV (data not shown).

Effects of associated comorbidity. Because the prevalence of comorbid diseases and the proportion of various treatment therapies at baseline differed in both groups of CHF (Table 1), we compared the values of Ang-1, Ang-2, tie-2, and VEGF with the past medical histories (ischemic heart disease, hypertension, cerebrovascular accident, diabetes, cholesterol level, smoking history) and various treatment therapies (beta blockers, angiotensin-converting enzyme inhibitors/angiotensin-1 receptor blockers, statin, warfarin, antiplatelet therapy) within the acute and chronic CHF groups. Using univariate analysis, high levels of Ang-2 were correlated with lower cholesterol levels ($r = -0.337$, $p = 0.003$) and (unsurprisingly) statin use ($r = -0.310$, $p = 0.005$). None of the markers were significantly influenced by age, gender, or past medical history (all p = NS, data not shown).

Stepwise multiple regression analyses. Using stepwise multiple regression analyses, cholesterol level and hydroxymethyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitor use, but not NYHA functional class, independently predicted Ang-2 levels in CHF ($p < 0.0001$ and $p = 0.005$, respectively). Only NYHA functional class independently predicted Ang-1 level ($p = 0.012$).

DISCUSSION

Published data on the role of the Ang/tie-2 system in cardiovascular diseases are limited. Most of our knowledge regarding the angiopoietins has come from oncology studies where angiogenesis is a prerequisite for tumor growth and metastasis. Indeed, Ang-2 has been shown to be a marker of a poor prognosis in breast cancer and non-small cell lung cancer (4,21). In the present paper, we describe a new assay for Ang-1 and, in addition, have demonstrated (for the first time in the literature) very abnormal levels of Ang-2 and tie-2, but normal Ang-1, in CHF. Vascular endothelial growth factor was marginally raised in the patients, suggesting that Ang-2 may be more relevant to the pathophysiology of this disease. In addition, there was a significant correlation between Ang-2 and tie-2.

That Ang-1 is not raised in CHF is consistent with currently held views that its secretion is not stimulated by hypoxia, as demonstrated in animal studies (13,14,22). Angiopoietin-1 has been shown to have antiapoptotic effects on endothelial cells through the activation of phosphatidylinositol 3′-kinase (15,23). This, in turn, stimulates the survival serine-threonine kinase, Akt, activation that is a requisite of survivin upregulation (24). The up-regulation of survivin protects endothelial cells from death-inducing stimuli. It is possible that the lack of stimulated Ang-1 secretion in CHF is partly responsible for the degree of endothelial damage observed in this condition (16).

The role of VEGF and the Ang in CHF remains unclear. Patients in chronic, stable CHF are frequently not hypoxic, yet plasma Ang-2 is elevated. Rather than just being purely
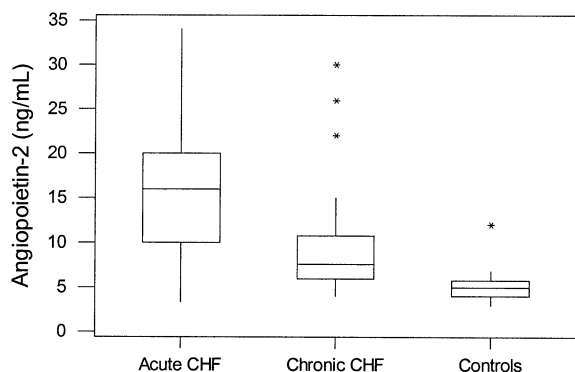

Figure 1. Angiopoietin-2 levels in acute congestive heart failure (CHF), chronic CHF, and controls. The **boxes** indicate the interquartile range with median value. The **asterisks** indicate outlying data points.
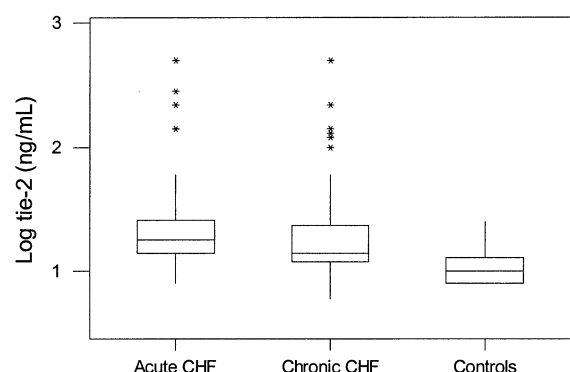

Figure 2. Plasma tie-2 levels in acute congestive heart failure (CHF), chronic CHF, and controls. The **boxes** indicate the interquartile range with median value. The **asterisks** indicate outlying data points.

mediators in angiogenesis, VEGF and Ang may, conceivably, play a role in endothelial homeostasis. Certainly, profound endothelial damage/dysfunction is associated with CHF as evident (for example) by elevated levels of von Willebrand factor (1,25). In addition, CHF is associated with (modestly) raised VEGF and now, Ang-2. Therefore, the VEGF and Ang-2 may mediate endothelial proliferation, not just in angiogenesis but also in endothelial repair. Indeed, VEGF has already been shown to stimulate endothelial proliferation and accelerate re-endothelialization after angioplasty-induced endothelial denudation in rabbit carotid arteries (26). Moreover, Ang-1 levels in CHF were not significantly different from controls, despite Ang-1-induced tie-2 phosphorylation being essential to initiate angiogenesis (10,27). However, there are also studies showing that Ang-2 and VEGF alone are sufficient for angiogenesis (12,28,29).

Several studies have shown that HMG-CoA reductase inhibitors have both proangiogenic and antiangiogenic effects. One showed a biphasic effect, being proangiogenic at low concentrations but antiangiogenic at high concentrations (30–32). In our study, there was a negative correlation between serum cholesterol level and Ang-2; HMG-CoA reductase inhibitor use was also associated with lower Ang-2. Clearly, this could be explained by the significantly greater use of HMG-CoA reductase inhibitor in chronic CHF compared with acute CHF. Unfortunately, we do not have serum concentrations of HMG-CoA reductase inhibitors available to draw any other conclusions, as neither their use nor Ang-2 correlated with outcome.

This study is limited by the single, global measurement of the angiopoietins and their soluble receptor. Therefore, we are unable to determine the levels of these mediators locally where angiogenesis occurs, nor the precise source or stimuli for these molecules in CHF. The Ang-2 messenger ribonucleic acid (RNA) expression in response to hypoxia is seen in microvascular endothelial cells and not macrovascular or lymphatic bovine endothelial cells (14). On the other hand, Ang-1 is secreted by periendothelial cells and acts in a paracrine manner. Hence, the plasma levels may not be representative of the cell biology of vasculogenesis, although several studies have shown that Ang-1 messenger RNA expression is not increased in a hypoxic environment. Secondly, we do not have data on angiopoietin messenger RNA levels in order to relate them to plasma levels. Indeed, we do not have direct evidence that angiogenesis, per se, actually occurs in CHF. We presume that, as there is hypoxia and elevated angiogenic factors, that angiogenesis takes place, but, as previously mentioned, these factors may also have a role in endothelial repair. While the lack of a clear and strong association with clinical indexes of ejection fraction and NYHA class (which would have been expected) raises the question of the value of these markers in a clinical setting, the abnormalities in the angiopoietin system may have implications for the (complex) pathophysiology of CHF. Ongoing prospective studies in our group are cur-

rently examining the relation of the angiopoietin system to etiology, treatments (e.g., angiotensin-converting enzyme inhibitors, beta-blockers, statins, and so on), and prognosis in CHF.

Conclusions. Elevated levels of Ang-2 (but not Ang-1) and its receptor tie-2 are present in both acute and chronic CHF patients that may, alongside VEGF, indicate a role for these angiogenic factors in the pathophysiology of CHF.

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