Endothelial Dysfunction and Damage in Congestive Heart Failure
Relation of Flow-Mediated Dilation to Circulating Endothelial Cells, Plasma Indexes of Endothelial Damage, and Brain Natriuretic Peptide

Aun Yeong Chong, MRCP; Andrew D. Blann, PhD; Jeetesh Patel, PhD; Bethan Freestone, MRCP; Elizabeth Hughes, MD; Gregory Y.H. Lip, MD

Background—Congestive heart failure (CHF) is associated with endothelial perturbation (as defined by flow-mediated endothelial-dependent vasodilation [FMD, an index of endothelial dysfunction], circulating endothelial cells [CECs, an index of endothelial damage], or plasma indexes of endothelial damage/dysfunction [eg, von Willebrand factor (vWf) and soluble thrombomodulin (sTM)]) and raised plasma levels of brain natriuretic peptide (BNP, a peptide hormone associated with left ventricular systolic dysfunction and prognosis). However, the relations between these parameters are unclear.

Methods and Results—To test the hypothesis that there is a relation between endothelial perturbation (defined by FMD, CECs, vWf, and sTM) and BNP in CHF, we studied these indexes in 30 patients with CHF who were compared with 20 age-matched control subjects. FMD, CECs, plasma vWf, and BNP levels (but not sTM) were all abnormal in patients with CHF. There were significant inverse correlations between FMD and vWf (P < 0.001), CECs (P < 0.002) and BNP (P = 0.006) as well as a positive correlation between CECs and vWf (P = 0.032). In multivariate analysis, BNP (P < 0.001) and FMD (P < 0.001) were both independently associated with CHF.

Conclusions—Ample evidence of endothelial cell damage/dysfunction in CHF cannot be fully explained by the variance in plasma BNP per se. Therefore, the routes by which these indexes influence the pathophysiology of CHF as well as predict adverse outcomes may be independent. (Circulation. 2004;110:1794-1798.)

Key Words: heart failure ■ endothelium ■ von Willebrand factor ■ endothelium-derived factors ■ natriuretic peptides

Considerable evidence indicates that congestive heart failure (CHF) is associated with abnormal endothelial function, assessed physiologically by impaired flow-mediated dilatation (FMD) and by increases in specific plasma indexes of endothelial damage/dysfunction, such as von Willebrand factor (vWf) and soluble thrombomodulin (sTM).

Quantification of circulating endothelial cells (CECs) is a relatively novel technique used to assess the disruption of endothelial integrity. Present in very low levels in “healthy” blood, raised numbers of CECs have been demonstrated in acute myocardial infarction, acute coronary syndromes, and critical limb ischemia and are probably the most direct evidence of severe endothelial damage. In cardiovascular disease, FMD has been shown to predict prognosis in hypertensive patients, whereas the data on sTM are conflicting.

Increased levels of vWf, on the other hand, are well established in cardiovascular disease and undoubtedly mark a poor prognosis in CHF, atrial fibrillation, and other diseases.

Brain natriuretic peptide (BNP) is a hormone secreted predominantly by the myocytes of the left ventricle in response to elevated wall stress. It has been shown to be a reliable diagnostic and prognostic tool in the management of CHF. Apart from the more widely recognized function of BNP (ie, natriuresis), there is growing recognition that BNP also acts on the endothelium, for example, in mediating vasodilation through nitric oxide (NO), possibly by influencing the activity of endothelial and inducible NO synthases (eNOS and iNOS, respectively), providing a link between natriuretic peptides and endothelial function. However, it is currently unclear whether these changes reflect other aspects of endothelial dysfunction.

The present status of the pathophysiology of vascular biology in CHF recognizes raised vWf (as a plasma index of endothelial damage/dysfunction) and impaired FMD (as a measure of endothelial dysfunction), although the relation between them is unknown. No data have been published on CECs in CHF, and available data on sTM are limited. The position of raised BNP in relation to endothelial indexes is also unclear. We therefore hypothesized the following: (1) that raised CECs and sTM are present in CHF, and, conse-
TABLE 1. Baseline Characteristics of Study Population

<table>
<thead>
<tr>
<th>Variable</th>
<th>Chronic CHF (n=30)</th>
<th>Control Subjects (n=20)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>65±12</td>
<td>64±9</td>
<td>0.640</td>
</tr>
<tr>
<td>Sex, M:F, n</td>
<td>19:11</td>
<td>6:14</td>
<td>0.021</td>
</tr>
<tr>
<td>LVEF, %*</td>
<td>31 (29–35)</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>NYHA class, n</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I–II</td>
<td>19</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>III–IV</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Comorbidity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IHD</td>
<td>23 (76.7)</td>
<td>0</td>
<td>...</td>
</tr>
<tr>
<td>HT</td>
<td>12 (40.0)</td>
<td>0</td>
<td>...</td>
</tr>
<tr>
<td>DM</td>
<td>12 (40.0)</td>
<td>0</td>
<td>...</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>4.4±1.3</td>
<td>5.9±0.9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Current smoking</td>
<td>5 (16.7)</td>
<td>1 (5)</td>
<td>0.214</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACEI/ARB</td>
<td>29 (96.7)</td>
<td>0</td>
<td>...</td>
</tr>
<tr>
<td>Spironolactone</td>
<td>6 (20.0)</td>
<td>0</td>
<td>...</td>
</tr>
<tr>
<td>β-Blocker</td>
<td>12 (40.0)</td>
<td>0</td>
<td>...</td>
</tr>
<tr>
<td>Warfarin</td>
<td>1 (3.3)</td>
<td>0</td>
<td>...</td>
</tr>
<tr>
<td>Statin</td>
<td>18 (60.0)</td>
<td>0</td>
<td>...</td>
</tr>
<tr>
<td>Nitrate</td>
<td>13 (43.3)</td>
<td>0</td>
<td>...</td>
</tr>
</tbody>
</table>

Data expressed as mean±1 SD, median (IQR) or n (%) unless stated; ... not estimated. NYHA indicates New York Heart Association classification (I, asymptomatic; II, symptoms at rest or minimal exertion; III–IV, symptoms at rest or minimal exertion); LVEF, left ventricular ejection fraction on M-mode echocardiography or Simpson’s method; IHD, ischemic heart disease (previous myocardial infarction or history of angina pectoris with positive exercise tolerance test or coronary disease on angiography); HT, hypertension; DM, diabetes mellitus; ACEI, angiotensin-converting enzyme inhibitor; and ARB, angiotensin receptor blocker.

*The median LVEFs are 31% (IQR 29% to 36%) for the 19 patients in NYHA classes I–II and 32% (25% to 35%) for the 11 patients in NYHA classes III–IV.

quently, (2) that alongside raised vWF and BNP levels, these indexes would correlate inversely with FMD. Finally, we intended, as a post hoc analysis, to determine which of these 5 research indexes was linked most closely and independently to CHF.

Methods

Subjects

We studied 30 patients with stable, chronic CHF, who were defined as being in a stable New York Heart Association (NYHA) class for at least 3 months, with no changes in drug therapy or admissions to hospital. All patients were in sinus rhythm and had documented left ventricular ejection fraction (LVEF) of <40% by M-mode echocardiography or Simpson’s method in the presence of regional wall motion abnormality. Patients were classified according to NYHA criteria, with NYHA I–II being mild symptoms and III–IV being moderate to severe symptoms. Patients were compared with 20 age-matched, healthy control subjects. Clinical characteristics of patients and healthy control subjects are summarized in Table 1.

Exclusion criteria were concomitant atrial fibrillation, acute coronary syndromes, infection or pyrexial illness, recent (<3 months) myocardial infarction or stroke, chronic and systemic illnesses (including renal failure, hepatic impairment, cancer, inflammatory connective tissue disease, inflammatory bowel disease, chronic obstructive airways disease), history of thromboembolism and the use of nonsteroidal antiinflammatory drugs (NSAIDs, except aspirin which was considered unethical to withdraw), oral steroids, and hormone replacement therapy. Heart failure primarily caused by valvular disease or pulmonary disease was also excluded. Healthy control subjects were recruited from among healthy hospital staff, spouses of patients, and subjects attending 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. The West Birmingham Research Ethics Committee approved the study protocol, and all patients gave written informed consent to the study.

FMD and Echocardiography

High-resolution ultrasound was used to assess changes in the diameter of the brachial artery, as previously described.13,14 Measurements were taken after the patient had rested in a supine position for 20 minutes in a quiet room. High-quality images were obtained by a single dedicated operator using a 10-MHz vascular ultrasound probe (GE Vingmed Ultrasound, System V). A longitudinal section of the brachial artery was scanned 5 to 10 cm above the antecubital fossa. The transducer remained in a fixed position relative to the patient’s arm throughout the procedure. Vessel diameter was assessed at end-diastole, demarcated by the onset of the R wave on the ECG trace, with 5 measurements over a short segment of the artery from leading edge to leading edge and averaged. After a baseline scan, a pneumatic cuff was placed at the level of the mid forearm and inflated to 250 to 300 mm Hg for 4.5 minutes. The second scan was performed 60 to 90 seconds after cuff release (peak changes during reactive hyperemia), and 15 minutes was allowed for vessel recovery. Subsequent scans of the brachial artery were done before and again at 4.5 minutes after the administration of sublingual glyceryl trinitrate (GTN; 400 μg spray) to assess endothelium-independent vasodilation. The repeat baseline scan is to take into account the possibility of a change in the resting tone so that the final change in vessel diameter is purely due to the effect of GTN.13 Scans were recorded on optical discs for later analysis by an independent investigator blinded to patient group. Interobserver and intraobserver variation was <10%. FMD and GTN-induced (endothelium-independent) dilation were estimated percent change in diameter relative to their respective baseline measurements.

Laboratory

Citrated and EDTA plasma was obtained from venous blood by centrifugation at 3000 rpm (1000g) for 20 minutes at 4°C. Aliquots of plasma were then stored at −70°C to allow batch analysis. vWF was measured by an established ELISA (Dako). STM was measured with commercially available ELISA kits (Diagnostica Stago). BNP was measured with a fully automated, 2-site sandwich immunoassay by direct chemiluminescent on EDTA plasma, using the ADVIA Centaur analyser (Bayer Diagnostics Ltd). The intra-assay coefficient of assays was <5%; interassay variation was <10%.

Four milliliters of blood for CECs was collected in a sodium fluoride tube and prepared for immunomagnetic separation within 1 hour and counted by a single observer under epifluorescence microscopy (Zeiss). Blood collection for CECs was collected last (after approximately 20 mL blood was collected for other analyses) to avoid the possibility of collecting endothelial cells dislodged as a consequence of venepuncture. The detailed methodology for capturing CECs and criteria for counting CECs in our unit has been previously described.3 Intra-assay (n=40) and interassay (n=20) coefficients of variation were <5% and <10%, respectively; the interobserver and intraobserver variations of the method in our laboratory were <5%. All laboratory work was performed in blinded fashion with regard to the identity of the samples.

Power Calculations

We have previously reported increased CECs in the plasma of 26 subjects with acute myocardial infarction and 33 with unstable angina compared with 13 with stable angina and 14 healthy control
TABLE 2. FMD, vWF, sTM, and CECs in Subjects With CHF and Control Subjects

<table>
<thead>
<tr>
<th>Variable</th>
<th>Chronic CHF</th>
<th>Control Subjects</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNP, pmol/L</td>
<td>28.8 (18.9–118.8)</td>
<td>5.3 (1.6–13.5)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>FMD, %</td>
<td>1.5 (0.0–3.5)</td>
<td>9.1 (5.3–15.2)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>EID, %</td>
<td>13.3 (5.0–21.0)</td>
<td>13.3 (9.5–22.9)</td>
<td>0.337</td>
</tr>
<tr>
<td>vWF, IU/dL</td>
<td>197±93</td>
<td>123±67</td>
<td>0.005</td>
</tr>
<tr>
<td>sTM, ng/mL</td>
<td>45.0 (16.8–80.1)</td>
<td>37.5 (25.0–47.5)</td>
<td>0.286</td>
</tr>
<tr>
<td>CECs, cells/mL</td>
<td>11.8 (4.6–18.4)</td>
<td>3.7 (1.3–7.2)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

EID indicates endothelium-independent (GTN-mediated) dilation. vWF expressed as mean±1 SD; FMD, EID, sTM, and CECs expressed as median (IQR).

P values from t test for vWF, Mann-Whitney test for BNP, FMD, EID, sTM, and CECs.

Subjects with an overall F statistic of 16, giving a probability value of <0.0001. We have also demonstrated a 3-fold increase in CECs in 20 patients with critical limb ischemia compared with 20 with intermittent claudication and 20 healthy control subjects (P<0.001). Consequently, we hypothesized similar levels and distribution in chronic CHF versus control subjects. Thus, with two groups, our power calculation required 20 subjects with CHF to have 3-fold higher CECs than the control group, generating a value of P=0.01. This target number of subjects (n=40) provides the power to detect a correlation coefficient of 0.3 at P<0.05 and 1–β=0.80. However, for additional confidence, we recruited 30 consecutive patients with CHF.

Statistical Analysis

Data were analyzed by the Shapiro-Wilks test to determine distribution. Normally distributed data are expressed as mean±SD. Data for LVEF, FMD, BNP, sTM, and CECs were not normally distributed and therefore are expressed as median (interquartile range, IQR). Baseline cross-sectional data between chronic CHF and healthy control subjects were analyzed by t test or Mann-Whitney test as appropriate. Correlations were performed by using Spearman’s rank correlation method. A 2-tailed probability value of <0.05 was considered statistically significant. Stepwise multiple regression analyses were used to determine the following: (1) which of vWF, sTM, CECs, and BNP were independently associated with FMD; and (2) which of these indexes were most closely associated with CHF.

Results

Results are presented in Table 2. In patients with CHF, there were (3-fold) higher levels of CECs (P<0.0001) but no difference in sTM compared with control subjects. As expected, there was raised BNP (approximately 5-fold, P<0.0001), poorer FMD (approximately 6-fold, P<0.0001), and raised vWF (approximately 1 SD, P=0.005). There was no significant difference in equivalent endothelium-independent (GTN-mediated) vasodilation. In the entire cohort of 50 subjects, there were modest inverse correlations between FMD and vWF (Spearman, r=-0.461, P=0.001), CECs (Spearman, r=-0.423, P=0.002) and BNP (Spearman, r=-0.419, P=0.006), as well as an expected positive correlation between CECs and vWF (Spearman, r=0.29, P=0.032) (Figures 1 through 3). Within the CHF group alone, only the correlation between FMD and vWF was significant (Spearman, r=-0.457, P=0.011). Acknowledging the likely confounder of diabetes, we performed analyses for this risk factor. FMD was significantly lower in the 12 patients with CHF with diabetes (mean±SD, −0.1±3.1%) compared with the 18 CHF patients free of diabetes (3.2±3.4, P=0.01). Even excluding the diabetic subgroup, FMD was significantly lower in patients with CHF without diabetes compared with control subjects (P<0.0001).

Multivariate Analysis

To determine those factors associated with FMD (as that index most abnormal in CHF compared with health), we entered indexes significantly different in univariate analysis (ie, vWF, CECs, BNP) into multivariate stepwise logistic and regression analyses. Here, vWF (P=0.320) and BNP (P=0.074) dropped out, leaving CECs (P=0.021) as the only significant independent predictor of FMD. Together, these 3 variables accounted for

Figure 1. Relation between FMD and circulating endothelial cells. Spearman r=−0.423; P=0.002.

Figure 2. Relation between FMD and vWF level. Spearman r=−0.461; P=0.001.
33.5% in the variation of FMD. We then determined which of the 4 indexes (vWf, CEC, BNP, and FMD) independently predicted CHF. In this analysis, both BNP ($P<0.001$) and FMD ($P=0.002$) were significant. Placing only FMD and BNP in a multivariate analysis, both were independently retained (both $P<0.001$), although stepwise analysis placed BNP most significantly, accounting for 49.2% of the variability of CHF/health. FMD alone accounted for 31.3% in the variability; BNP and FMD together accounted for 66.1% of the variance.

**Discussion**

Endothelial integrity can be assessed by a number of techniques, the longest established being release of vWf, with raised levels previously reported in CHF. Since then, FMD has been developed as a more “physiological” method of assessing the endothelium, centering on endothelium-dependent vasodilation in response to shear stress and therefore an indirect measure of eNOS activity. Using strain-gauge plethysmography, it has been reported that the impairment of endothelium-dependent vasodilation was near maximum even in mild CHF. This “plateau effect” could explain the lack of relation between FMD and the severity of CHF. Indeed, impaired endothelium-dependent vasodilation may explain the abnormal vasoconstriction that is a hallmark of CHF, and the detrimental effect of NSAIDs in CHF may extend beyond that as the result of salt and water retention. More recently, increased numbers of CECs in the blood unequivocally demonstrate severe endothelial perturbation in cardiovascular disease, but levels in CHF have not been previously reported.

The inverse correlation between FMD and vWf in the present study of patients with CHF is mirrored in a substudy of hypertensive patients in the Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT) and also in type 2 diabetic patients with treated hypertension. This is consistent with current views on the function of the endothelial monolayer in mediating vasodilation in response to increased shear stress with raised vWf being an established marker of endothelial damage/dysfunction. Furthermore, the current observed (respectable) inverse relation between FMD and CECs lends further support to our viewpoint that CECs are desquamated, damaged, or dysfunctional endothelial cells. An increased number of CECs raises the possibility that not only is the endothelial monolayer dysfunctional, but some parts may in fact be denuded of vascular endothelial cells. Akin to early in vitro observations of the effect of endothelium denudation on endothelium-dependent vasodilation, we suggest that our data provide indirect in vivo proof of that concept in humans. Furthermore, this suggests that endothelial damage in CHF is generalized. To be able to explain the significant relations between FMD, vWf, and CECs, we have to accept that FMD measured in only a very short segment of the brachial artery is representative of the endothelium in its entirety. Indeed, the endothelium should be recognized as an organ in its own right and in this respect, targeted specifically as a therapeutic measure. Endothelial denudation also exposes the underlying prothrombotic collagen that forms the subendothelial layer, and the resultant activation of the coagulation system may explain the excess risk of thromboembolism associated with CHF. This coupled with raised vWf that facilitates platelet adhesion to other platelets and the endothelium further exacerbates the problem in CHF.

The other inverse relation between plasma BNP and FMD is interesting, as neither BNP nor FMD correlated with LVEF or NYHA class in our study, possibly because of the small number of subjects. However, both were independently selected in multivariate analysis of factors linked to CHF, providing approximately 49% and 31% variability, respectively. The two markers seem likely to have some interrelation, as together they provide 66% of the variability in CHF and have a respectable significant correlation. Thus far, there is only conflicting evidence from a few studies of such a possible relation, some showing upregulation of eNOS and iNOS by ANP and BNP and others downregulation of tumor necrosis factor-α (TNF-α) and, indirectly, iNOS by ANP. Although strictly speaking, the latter studies were performed using ANP, both ANP and BNP appear to have identical functions. Therefore, whereas upregulation of eNOS and iNOS should improve FMD as shown by exogenous administration of BNP, albeit in healthy individuals, downregulation of TNF-α and consequently iNOS may impair FMD further. Indeed, in patients with CHF, iNOS has been shown to play a significant role in resistance vessel tone and endothelium-dependent vasodilation. Thus, it is plausible that high plasma BNP may worsen FMD in patients with CHF.

**Study Limitations**

We acknowledge that the number of patients in our present study was small. However, this was intended to be a pilot study, as there are no data on CECs or its relation to other markers of endothelial perturbation. The study was not designed to evaluate the effect of dosages of various medications and certainly was inadequately powered to do so. Patients who were taking NSAIDs other than low-dose aspirin were excluded from the present study because animal experiments suggest that endothelial-derived prostanoids may play a role in FMD as well; however, because of ethical considerations, we did not discon-
tinue low-dose aspirin because the majority of the patients with CHF either had coronary artery disease or were at significantly high risk of a cardiovascular event. Even if we assume that aspirin did attenuate some of our research indexes, we would expect an even greater difference between patients with CHF and control subjects.

Conclusions

In conclusion, we have confirmed raised vWF, raised BNP, and impaired FMD in CHF. Notably, all 3 of these endothelial assessments significantly intercorrelated. Our first novel finding is of raised CECs but no change in sTM in this disease. The second contribution of our data is a correlation between BNP and FMD, but both remained as significant independent (statistical) predictors of the presence of CHF. This implies to us that the factors responsible for the increase in BNP in CHF may be independent of those responsible for endothelial damage/dysfunction. Therefore, the routes by which these indexes influence the pathophysiology of CHF as well as predict adverse outcomes may be independent.

Acknowledgments

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