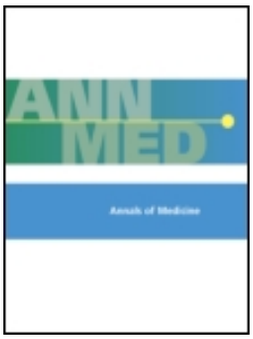

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ORIGINAL ARTICLE

Angiogenic factors in atrial fibrillation: A possible role in thrombogenesis?

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Abstract

Background. The precise pathophysiological processes underlying the prothrombotic or hypercoagulable state in atrial fibrillation (AF) remain uncertain. We hypothesized a relationship between abnormal endothelial damage/dysfunction, coagulation, and angiogenic factors, thereby contributing to increased thrombogenicity.

Methods. Plasma levels of von Willebrand factor (vWF, an index of endothelial damage/dysfunction) and tissue factor (TF, an index of coagulation), as well as the angiogenic factors, vascular endothelial growth factor (VEGF), angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2), were measured by enzyme-linked immunosorbant assay (ELISA) in 59 chronic AF patients. Data were compared to 40 age- and sex-matched healthy controls in sinus rhythm.

Results. Plasma vWF, VEGF and Ang-2 were significantly higher in AF patients compared to healthy controls ($P=0.005$, $P=0.0055$ and $P<0.0001$ respectively) but there were no significant differences in plasma Ang-1 or TF levels between the two groups ($P=0.925$ and $P=0.121$ respectively). Significant correlations were found between VEGF and vWF levels (Spearman, $r=0.262$, $P=0.011$) and between VEGF and Ang-2 ($r=0.333$, $P=0.001$).

Conclusions. Raised VEGF in association with Ang-2 and vWF may reflect a link between abnormal endothelial damage/dysfunction and angiogenic factors. These may act together to alter TF expression and endothelial integrity, thereby contributing to the prothrombotic state in AF.

Key words: *Angiopoietin, endothelium, tissue factor, vascular endothelial growth factor*

Introduction

Atrial fibrillation (AF) is associated with thromboembolic tendency and is a leading cause of stroke and thromboembolism (1), probably due to abnormalities in haemostasis, platelets and indices of endothelial damage/dysfunction (2) that seem independent of associated structural heart disease or underlying aetiology of AF (3). However, the precise pathophysiological processes underlying the prothrombotic or hypercoagulable state in atrial fibrillation (AF) remain uncertain.

Endothelial damage/dysfunction may be central to the pathogenesis of the prothrombotic state in AF (4). Indeed, indices of endothelial damage/dysfunction, such as plasma von Willebrand factor (vWF) are consistently abnormal in AF (5–7) and vWF has even been associated with stroke risk stratification and adverse outcomes in AF patients (8,9). As well as vWF, other endothelial measures such as forearm plethysmography, nitric oxide production, plasma E-selectin and soluble thrombomodulin are also abnormal in AF (10–12,6).

There are many possible indices of angiogenesis. For example, vascular endothelial growth factor (VEGF) is one such marker, and is a potent endothelium-specific angiogenic factor, which acts to promote endothelial cell survival, proliferation and migration (13). The VEGF gene and protein are both strongly expressed in actively angiogenic areas, and it exerts its biological effects through binding its

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high-affinity tyrosine kinase receptors Flt-1 and Flk-1/KDR. VEGF is thought to play an essential role in angiogenesis by increasing endothelial monolayer permeability (14), but in addition, this molecule also acts as a pro-inflammatory cytokine by inducing the expression of adhesion molecules that bind leucocytes to endothelial cells (15), as well as increasing tissue factor expression and pro-coagulant activity (16). The importance of tissue factor (TF) is its powerful procoagulant activity on forming a complex with factor VII(a), and it is widely regarded as the physiological trigger to blood clotting as it drives thrombin formation (17). Importantly, VEGF has been shown to be raised in AF (18,19). Raised VEGF has been associated with increased TF levels in AF, linking angiogenesis (or VEGF) to thrombogenesis (19), in keeping with data from cancer biology (20).

More recently, oncology research has identified other growth factors, such as those of the angiopoietin family, that appear to have a major role in angiogenesis and metastasis (21–23). The roles of two members of this family, angiopoietin-1 and angiopoietin-2 (Ang-1 and Ang-2) are becoming understood, and may act alongside VEGF. VEGF is also known to increase Ang-2 levels (24), and can also have an influence on TF activity. Indeed, the balance of Ang-1 and Ang-2 levels has been proposed to have an important homeostatic role via their respective agonist and antagonistic actions on Tie-2 (an endothelial receptor tyrosine kinase). When the balance is in favour of Ang-1, endothelial stability is promoted – whereas when the actions of Ang-2 predominate, endothelial destabilization, new vessel sprouting and facilitation of the actions of other vascular cytokines such as VEGF are favoured.

We hypothesised a link between abnormal endothelial damage/dysfunction and angiogenic factors, which may act together to alter tissue factor expression and endothelial integrity, thereby contributing to increased thrombogenicity. We tested this hypothesis in a cross-sectional study of 59 chronic AF patients and 40 controls in sinus rhythm, in whom levels of vWF and TF, as well as the angiogenic factors, VEGF and Ang-1&2, were measured by ELISA.

Key messages

- The precise pathophysiological processes underlying the prothrombotic or hypercoagulable state in atrial fibrillation (AF) remain uncertain. Abnormalities of angiogenesis and endothelial damage/dysfunction may have a role.
- In this study we found abnormal vascular endothelial growth factor (VEGF, associated with angiogenesis) in association with angiopoietin-2 (another index of angiogenesis) and vWF (an index of endothelial damage/dysfunction) in AF, which may reflect a link between an abnormal endothelium and angiogenic factors.
- These abnormalities may act together to alter tissue factor expression and endothelial integrity, thereby contributing to the prothrombotic state in AF.

Methods

We studied 59 outpatients with chronic AF attending our specialist clinic. All patients had a history of AF of >6 weeks, and no evidence of valvular heart disease or left ventricular dysfunction (by clinical examination and echocardiography). Exclusion criteria also included previous myocardial infarction, documented coronary artery disease at angiography, diabetes mellitus, a history of an acute cerebrovascular event (within 1 month), malignancy, connective tissue or inflammatory disease, acute or chronic infection, hepatic impairment and renal impairment.

Results were compared to 40 age- and sex-matched healthy control subjects, recruited from hospital staff and relatives or friends of patients who attended hospital. Healthy controls had no history of diabetes, hypertension, neoplastic, connective tissue or cardiovascular disease and all subjects underwent careful screening with baseline bloods, blood pressure measurement, electrocardiography and echocardiography. All healthy controls were 'healthy' by virtue of normal clinical history and examination, as well as basic blood screening tests, electrocardiogram (ECG) and echocardiography. The study was conducted in accordance with the Declaration of Helsinki and following the approval of the local research ethics committee. Written informed consent was obtained from all participants.

Laboratory

Venous blood was obtained from subjects in the fasted state and, where possible, >8 hours following any medication. Citrated plasma samples were collected and immediately placed on ice for a maximum of 20 minutes before processing. Platelet-free citrated plasma was obtained from venous blood by centrifugation at 3000 rpm for 20 minutes at 4°C. Plasma was then aliquoted and frozen at −70°C for subsequent batch analysis.

Plasma vWF was measured using an established enzyme-linked immunosorbant assay (ELISA) (Dako, Glostrup, Denmark). Tissue factor (pg/mL) was measured by ELISA using reagents and standards from Axis-Shield (Dundee). VEGF, Ang-1 and Ang-2 (ng/mL) were all measured by ELISA using reagents from R&D systems (Abingdon, Oxfordshire, UK). Intra-assay coefficients of variation for all ELISAs were $<5\%$ and interassay variances $<10\%$.

Power calculations and statistical analysis

We have previously reported plasma Ang-2 to be significantly raised (by 0.5 SD) in 40 patients with chronic congestive heart failure compared to 17 healthy controls ($P<0.001$) (25). In the present study, we set our power calculation at a minimum of 32 subjects per group to enable us to detect a difference of one standard deviation with $1-\beta=0.8$ and $P<0.05$ between groups.

Continuous data were subjected to the Anderson-Darling test to assess distribution, and are expressed as mean $\pm$ standard deviation (SD) or median and inter-quartile range (IQR) as appropriate. Differences between the patient group and controls were analysed by *t*-testing or Mann-Whitney tests, as appropriate. Categorical data were compared using chi-squared test. Correlations were performed using Spearman's rank correlation method. Multivariate analysis was performed by stepwise multiple regression analysis using research indices as the dependent variable(s) and clinical variables (e.g. age, gender, hypertension, etc.) and the presence/absence of AF as predictors. A two-tailed P value <0.05 was considered statistically significant.

Results

Demographic and clinical data for patients and controls are summarized in Table I. The median duration of AF (defined from the time of first electrocardiographic confirmation) was 6.5 months (IQR 3.75–24) and of note, 40 of the AF group were already receiving warfarin, 13 were on aspirin and 6 had not yet started antithrombotic therapy at the time of blood sampling, although antithrombotic therapy has not been noted to alter our research indices (3).

Results are summarized in Table II. In patients with AF there were, as expected, higher plasma

Table I. Demographic and clinical data for AF cases and healthy controls.

Variable	Controls (n=40)	AF (n=59)	P
Patient demography			
Mean age (years)	64 ($\pm$9)	65 ($\pm$8)	0.333
Male, *n* (%)	17 (43%)	31 (52%)	0.369
Current smokers, *n* (%)	4 (10%)	2 (3%)	0.169
Co-morbidity			
Hypertension	-	33 (55%)	-
Hyperlipidaemia	-	5 (8%)	-
Previous CVA	-	7 (12%)	-
Treatment			
Aspirin	-	13 (22%)	-
Warfarin	-	40 (67%)	-
Digoxin	-	21 (35%)	-
Amiodarone	-	4 (7%)	-
Beta-blocker	-	26 (43%)	-
Calcium channel blocker	-	14 (23%)	-
ACE inhibitor/ARB	-	10 (17%)	-
Diuretic	-	21 (35%)	-
Statin	-	5 (8%)	-
Clinical measurements			
Systolic BP (mmHg)	134 ($\pm$20)	137 ($\pm$19)	0.488
Diastolic BP (mmHg)	79 ($\pm$8)	80 ($\pm$11)	0.629
Total Cholesterol (mMol/L)	5.6 ($\pm$0.9)	5.3 ($\pm$1.0)	0.070

Results are expressed as numbers and percentages, as mean ($\pm$standard deviation) or median (interquartile range). Chi-squared was used for categorical data and two sample *t*-test and Mann-Whitney tests were used as appropriate for parametric and non-parametric continuous variables. CVA=cerebrovascular accident; ACE=angiotensin converting enzyme; ARB=angiotensin 2 receptor blocker; BP=blood pressure.

Table II. Plasma levels of vWF, tissue factor, VEGF, angiopoietin-1 and angiopoietin-2 in AF patients in comparison to healthy controls.

	Healthy controls	AF patients	*P*-value
vWF (iU/dL)	118 (±60)	157 (+71)	0.005
TF (pg/mL)	455 (38–1100)	750 (150–1800)	0.121
VEGF (pg/mL)	50 (10–135)	110 (40–540)	0.0055
Ang-1 (ng/mL)	5 (2.2–7.4)	4 (2.5–12.5)	0.925
Ang-2 (ng/mL)	2 (1.5–3.6)	5.8 (2.6–8.6)	<0.0001

Results are expressed as numbers and percentages, as mean (±standard deviation) or median (interquartile range) with two sample *t*-test or Mann-Whitney test used as appropriate. vWF=von Willebrand factor); TF=tissue factor; VEGF=vascular endothelial-derived growth factor; Ang-1=angiopoietin 1; Ang-2=angiopoietin 2.

levels of vWF compared to healthy controls ($P=0.005$). VEGF and Ang-2 were also significantly raised in AF ($P=0.0055$ and $P<0.0001$ respectively). There was, however, no significant difference in Ang-1 or TF levels in comparison with control subjects ($P=0.925$ and $P=0.121$ respectively).

Effects of age, co-morbidity and concomitant warfarin or aspirin use in AF patients

There were no significant differences in vWF, TF, VEGF, Ang-1 or Ang-2 levels between AF patients when separated into those above and below the median age of 65 (IQR 64–68) years old (Table III). There were also no differences in research indices in those patients with AF who also had a diagnosis of hypertension when compared to those with no past medical history of hypertension (Table IV), and AF patients with other co-morbidities known to influence angiopoietin levels such as diabetes or known ischaemic heart disease (angina or prior myocardial infarction) were excluded from this study from the outset.

Warfarin use in the AF group did not influence either vWF plasma levels or plasma levels of the other endothelium-related indices, with no significant differences between those patients on and off warfarin treatment at the time of sampling (Table V). Similarly the use of aspirin had no influence on plasma vWF, VEGF, Ang-1, Ang-2 or TF (data not shown).

Correlations and multivariate analyses

In the entire cohort, there were weak but significant correlations found between vWF and VEGF levels (Spearman $r=0.262$, $P=0.011$), and VEGF and Ang-2 ($r=0.333$, $P=0.001$). Correlations of the

Table III. Difference in research indices in the AF group with age (group divided into those above and below the median age of 65).

	Age<65 years ($n=30$)	Age≥65 years ($n=29$)	*P*-value
vWF (iU/dL)	154 (±68)	160 (±76)	0.741
TF (pg/mL)	850 (318–1950)	600 (13–1600)	0.1622
VEGF (pg/mL)	125 (42–788)	100 (32–365)	0.3231
Ang-1 (ng/mL)	3.1 (2.05–12.75)	4.8 (2.75–12.0)	0.3753
Ang-2 (ng/mL)	5.8 (2.3–8.75)	5.8 (2.75–9.25)	0.5955

Results are expressed as mean (±standard deviation) or median (interquartile range) with two sample *t*-test or Mann-Whitney test used as appropriate. vWF=von Willebrand factor; TF=tissue factor; VEGF=vascular endothelial-derived growth factor; Ang-1=angiopoietin 1; Ang-2=angiopoietin 2.

Table IV. Difference in research indices in the AF group dependent on a pre-existent diagnosis of hypertension.

	No hypertension ($n=16$)	Hypertension ($n=33$)	*P*-value
vWF (iU/dL)	171 (±73)	146 (±69)	0.200
TF (pg/mL)	750 (250–2075)	850 (63–1700)	0.854
VEGF (pg/mL)	155 (30–315)	100 (40–925)	0.982
Ang-1 (ng/mL)	3.0 (2.0–16.5)	4.3 (2.5–8.5)	0.664
Ang-2 (ng/mL)	5.8 (2.7–8.75)	5.8 (2.45–9.0)	1.000

Results are expressed as mean (±standard deviation) or median (interquartile range) with two sample *t*-test or Mann-Whitney test used as appropriate. vWF=von Willebrand factor; TF=tissue factor; VEGF=vascular endothelial-derived growth factor; Ang-1=angiopoietin 1; Ang-2=angiopoietin 2.

Table V. Difference in research indices in the AF group dependent on whether on or not the patient was on warfarin at the time of blood sampling.

	No warfarin (n=19)	warfarin (n=40)	P-value
vWF (iU/dL)	165 (±69)	154 (±73)	0.591
TF (pg/mL)	1050 (250–2600)	725 (150–1350)	0.346
VEGF (pg/mL)	120 (40–9500)	100 (31–240)	0.445
Ang-1 (ng/mL)	6.0 (2.5–16.0)	3.3 (2.5–9.9)	0.167
Ang-2 (ng/mL)	7.5 (2.8–10)	4.8 (2.5–8.0)	0.146

Results are expressed as mean (±standard deviation) or median (interquartile range) with two sample t-test or Mann-Whitney test used as appropriate. vWF=von Willebrand factor; TF=tissue factor; VEGF=vascular endothelial-derived growth factor; Ang-1=angiopoietin 1; Ang-2=angiopoietin 2.

plasma levels of vWF, VEGF and Ang-2 with clinical variables (AF, sex, age hypertension, current smoking and prior cerebrovascular accident) and with the use of concurrent medication (listed in Table I), showed a significant association between vWF and AF (r=0.247, P=0.007), but no significant positive correlations with the other clinical indices or treatment listed. VEGF levels showed significant correlations with presence of AF (r=0.236, P=0.005) and previous CVA (r=0.239, P=0.019). Ang-2 levels correlated with AF (r=0.425, $P<0.0001$) and previously diagnosed hypertension (r=0.259, P=0.02).

To determine those factors independently associated with plasma vWF, VEGF and Ang-2, we used stepwise multiple regression analyses. The presence of AF was the only independent predictor of vWF levels (P=0.008) and was also the only independent positive predictor of Ang-2 ($P<0.001$) levels. The presence of AF just failed to reach significance for independently predicting VEGF levels (P=0.053), and CVA was not associated with VEGF on multiple regression analysis (P=0.784).

Discussion

In this study we have shown that Ang-2 is increased in AF in association with VEGF and vWF, thus demonstrating alterations in the balance of angiogenic factor expression in these patients. Furthermore, correlations between vWF and VEGF and between VEGF and Ang-2 would indicate a connection (by a common drive, origin or pathophysiological process) that links their expression.

Raised vWF in AF is consistent with numerous previous studies (2,5–7) and is almost certainly a reflection of endothelial damage/dysfunction in association with this arrhythmia, especially within the left atrium (26). The increased expression of vWF in the endocardium can be associated with enlarged left atrial dimensions in mitral valvular disease or increased myocyte diameters in the underlying myocardium, as well as platelet adhesion/aggregation on the endocardium (27). Indeed, the increased vWF in the endocardium may be a local predisposing factor for thrombogenesis in overloaded human atrial appendage (28). Theories as to the cause of endothelial damage/dysfunction in AF vary, but we postulate these could be associated with altered shear stresses to the vascular endothelium, either localized to the atrium or systemically due to a lack of regular pulsatile laminar flow (29,30). Changes in shear stress in AF may influence adhesion molecule expression and lead to a down-regulation in endothelial nitric oxide synthase expression and therefore endothelial cell nitric oxide production. These could be the first steps in a cascade of events with resultant platelet aggregation and adhesion, leucocyte adhesion, inflammatory cytokine release, further endothelial damage and vWF release into the circulation, thus promoting thrombus formation. The presence of other cardiovascular risk factors would, of course, compound this by further causing endothelial dysfunction therefore escalating the drive for thrombus formation in AF. Alternatively, alterations in endothelial indices may simply reflect associated vascular disease, although we did try to minimize the influence of this by exclusion of patients with known coronary or peripheral artery disease from our study.

Although there is no obvious requirement for angiogenesis in AF, we found VEGF levels again to be raised in this cohort, consistent with previous studies (18,19). In the current study, efforts were made to exclude other co-morbidities that have been associated with angiogenesis (such as diabetes mellitus or documented coronary artery disease), which would have confounded our results. Although patients with treated cardiovascular risk factors (hypertension and hyperlipidaemia) were included in our study group, both mean blood pressure and serum total cholesterol levels were comparable in

cases and controls and therefore should not have influenced our results.

In this study we have again shown the association of raised VEGF levels with AF. The drive for this is uncertain, but left atrial enlargement has been postulated to play a role, as mechanical stretch has been shown to induce rapid secretion of VEGF by cultured rat cardiac myocytes *in vitro* (31) and similarly smooth muscle cells when stretched exhibit up-regulation of hypoxia-inducible factor-1 (HIF-1)α, which in turn increases the expression of VEGF (as VEGF is the target gene for HIF-1) (32). In the former, the increased expression of VEGF has been suggested to be a response to relative tissue hypoxia, with a role in the protection of cardiac myocytes from tachyarrhythmia-induced stress and improvement of coronary microcirculation. However, generalized tissue hypoxia and angiogenesis are not features of AF, and an alternative explanation that we would put forward is that of a rise in VEGF being part of a maladaptive response to endothelial perturbation that acts to perpetuate rather than fix the problem. Another possible source of VEGF may be activated platelets (13), which may be implicated in the hypercoagulable state of AF (2).

We have previously suggested that both VEGF and Ang-2, rather than being angiogenic in congestive heart failure patients, may be necessary for endothelial cell repair and cell turnover and their increased secretion may be stimulated by endothelial damage or dysfunction (25). Indeed, increased VEGF levels may have a similar role in AF, and could promote the hypercoagulable state by increasing endothelial cell expression of adhesion molecules such as E-selectin, thereby encouraging leucocyte and platelet adhesion and activation, and could also act to increase endothelial tissue factor expression and procoagulant activity. In the present study, plasma VEGF levels also correlated with Ang-2 levels, in keeping with data showing that VEGF may increase Ang-2 expression (24). However, the relationship between vWF and Ang-2 can also be explained by the recent finding that they are co-secreted by endothelial cells as they are contained within a subset of Weibel Palade bodies (33).

Whatever the mechanism of Ang-2 increase, we do know that Ang-1 and Ang-2 are natural co-antagonists in that they compete for the same active binding site on Tie-2 (34,35) so although Ang-1 levels are not depressed in our patient group, with the balance in favour of Ang-2 we may assume that the actions of Ang-1 are inhibited. If this were the case, the raised Ang-2 levels in AF would be of relevance in that they would result in a loss of negative regulation of VEGF-induced tissue factor activity by Ang-1, and although TF levels were not significantly increased in this particular study group, we have previously demonstrated raised TF plasma levels in AF compared with healthy controls (19).

Therefore, rather than promoting angiogenesis *per se*, we suggest that an imbalance of Ang-1 and -2 could act to disrupt endothelial integrity, and increases in Ang-2 in association with increased VEGF could increase TF expression and procoagulant activity therefore playing an integral role in generation of the prothrombotic state. Indeed, the link between alterations in angiogenic factors and thrombogenicity proposed in atherosclerotic disease (36) may also be relevant in the setting of AF. The lack of influence of VEGF and Ang-2, on promoting new vessel formation would have to be further explored, but we speculate could be explained by concurrent suppression of the signalling pathways to angiogenesis. In AF, this could be explained by down-regulation of endothelial nitric oxide synthetase (eNOS) by reduced shear stress, rather than increased VEGF causing eNOS up-regulation, thereby disrupting the usual angiogenic signalling pathway of VEGF (37). Atrial natriuretic peptide (ANP), which is known to be raised in AF (38), has been shown to interrupt VEGF signalling via natriuretic peptide clearance receptor specific ligand (NPRC) and guanylate cyclase (GC) receptors (39). Therefore alterations in angiopoietin expression associated with concurrent increases in VEGF levels could be acting to increase TF without promoting angiogenesis.

This study is, of course, limited by its cross-sectional design and only allows us to explore novel associations therefore no causality is implied. The influence of co-morbidities such as hypertension and hyperlipidaemia, although treated, cannot be discounted. Additionally as there is evidence for alterations in angiogenic factor expression in cerebral infarction and ischaemia (40,41), the influence of previous cerebrovascular events also cannot be discounted, and although acute strokes (within 1 month) are excluded, it is acknowledged that this may be contributory to our findings.

We conclude that the raised VEGF levels in association with increases in Ang-2 and vWF demonstrated by this study may reflect a link between abnormal endothelial damage/dysfunction and angiogenic factors, which may act together to alter TF expression and endothelial integrity, thereby contributing to the prothrombotic state in AF. Indeed in light of the recently published finding that Ang-2 co-localizes with vWF in Weibel Palade

bodies and of their co-export from stimulated endothelial cells (33), our study would support a functional role for Ang-2 alongside vWF in vascular haemostasis in AF, or at the very least shows Ang-2 as yet another marker of endothelial perturbation in AF.

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