SCIENTIFIC REPORT

The angiopoietin/Tie-2 system in proliferative sickle retinopathy: relation to vascular endothelial growth factor, its soluble receptor Flt-1 and von Willebrand factor, and to the effects of laser treatment

J S Mohan, P L Lip, A D Blann, D Bareford, G Y H Lip

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Aim: To determine plasma levels of angiopoietin-1 and angiopoietin-2 (Ang-1, Ang-2), their soluble receptor Tie-2, vascular endothelial growth factor (VEGF), its soluble receptor Flt-1 (as indices of angiogenesis), and von Willebrand factor (vWf, marking endothelial damage/dysfunction) in sickle cell disease (SCD) patients with proliferative sickle retinopathy (PSR), with non-proliferative retinopathy (NPR), or no retinopathy (NR) and in control subjects with normal haemoglobin (AA subjects). In addition, to determine changes with panretinal laser photocoagulation (PRP) therapy.

Methods: Research indices were measured (ELISA) in 24 SCD patients who had PSR, 16 with NPR, 16 with NR, and from 23 AA subjects. Eight patients received PRP therapy and plasma was obtained before laser treatment and at 6 months after the last PRP session.

Results: Ang-1, Ang-2, VEGF, and vWf (but not Tie-2 or sFlt-1) were raised in SCD patients compared to AA subjects ($p<0.01$) but there were no differences among the three SCD subgroups. Significant correlations were between Ang-1 and VEGF, Ang-1 and Tie-2, and VEGF and sFlt-1 in patients with SCD ($r=0.67$–0.88). Plasma Ang-2, VEGF, sFlt-1, and vWf levels did not change, but Ang-1 fell and Tie-2 rose significantly following PRP therapy.

Conclusions: SCD patients have raised plasma angiopoietins (Ang-1, Ang-2), VEGF, and vWf compared to AA subjects. These indices did not differ according to severity of retinopathy and only limited changes occurred following PRP. The elevated growth factor levels in SCD may have obscured any association with retinopathy.

In sickle cell disease (SCD), a group of inherited disorders of haemoglobin where pathology is caused by sickle (HbS) haemoglobin, the major pathophysiological basis of the morbidity and mortality is microvascular vaso-occlusion. Vaso-occlusion in the microcirculation of the peripheral retina in SCD results in ischaemia, a response to which is a sequence of pathological vascular events leading to proliferative sickle retinopathy (PSR), eventually causing visual loss in 10–12% of eyes.[1 2] However, the determinants of PSR progression or autoinfarction are unknown.[3]

Vascular endothelial growth factor (VEGF), a potent, secreted growth factor that promotes angiogenesis, is a possible stimulus for retinal proliferation, as its expression is upregulated by hypoxia.[4] High intraocular VEGF is found in patients with active intraocular neovascularisation,[5 6] supporting the view of a role in vasoproliferation/neovascularisation. Changes in both plasma and intraocular VEGF levels have been related to laser treatment.[5 7] VEGF interacts with endothelial cells via membrane spanning receptors Flt-1 and KDR and the role of Flt-1 in embryonic vasculogenesis and adult angiogenesis and its association with several diseases has been clearly established.[8]

Angiopoietin-1 and angiopoietin-2 (Ang-1, Ang-2), members of another family of vascular growth factors, interact with the endothelial cell specific tyrosine kinase receptor Tie-2.[9] Ang-1 acts via the Tie-2 receptor to remodel primitive vessels and help maintain and stabilise the mature vessels by promoting interaction between endothelial cells and surrounding support cells.[10 11] Ang-2, conversely, leads to destabilisation of vessels and dissociation of pericytes, and is upregulated by hypoxia and angiogenic cytokines, including VEGF[12 13] and in pathological angiogenesis associated with tumours[10 11] and choroidal neovascularisation associated with age related macular degeneration.[14] Thus, the precise balance of VEGF and the angiopoetin/Tie-2 system is essential for modulating growing vessels and maintaining the integrity of existing vessels, thereby determining whether vessels proliferate and become leaky.

Abnormally raised levels of VEGF have been reported in SCD[15] but any role in associated eye disease is unclear. We therefore hypothesised abnormal Ang-1, Ang-2, sFlt-1, and sTie-2 in SCD, measuring VEGF as an effective positive control and vWf as an index of endothelial damage/dysfunction. We further hypothesised a graded increase in these markers in SCD patients with no retinopathy (NR), others with non-proliferative retinopathy (NPR), and others with proliferative sickle retinopathy (PSR). To test these hypotheses, we undertook a cross sectional study. Finally, we hypothesised that these indices fall after treatment with panretinal laser photocoagulation (PRP), and conducted a longitudinal study, measuring our research indices before and 6 months after laser treatment.

PATIENTS AND METHODS

Patients with SCD who attended the Sickle Cell and Thalassemia (SCAT) Centre were recruited. Diagnosis (HbSS or HbSC) was proved using routine high performance liquid chromatography (HPLC). Patients were excluded if they had previously received laser treatment for proliferative retinopathy or had eye surgery, were receiving regular red cell exchange tranfusion, had blood tranfusion within the

Abbreviations: Ang, angiopoietin; HbS, sickle haemoglobin; HPLC, high performance liquid chromatography; NPR, non-proliferative retinopathy; NR, no retinopathy; PRP, panretinal laser photocoagulation; PSR, proliferative sickle retinopathy; SCD, sickle cell disease; VEGF, vascular endothelial growth factor; vWf, von Willebrand factor

Table 1 Plasma levels of angiopoietin-1 (Ang-1) angiopoietin-2 (Ang-2), the soluble angiopoietin receptor Tie-2 (Tie-2), vascular endothelial growth factor (VEGF), its soluble receptor Flt-1, and von Willebrand factor (vWf) in patients with sickle cell disease (SCD) and subjects with normal haemoglobin (AA)

	AA subjects (n = 23) (IQR)	SCD patients (n = 56) (IQR)	p Value
Raw data			
Ang-1 (ng/ml)	0.5 (0.5–2.5)	2.2 (1.0–11.4)	0.0004
Ang-2 (ng/ml)	1.3 (1.0–2.0)	5.1 (2.3–7.7)	<0.001
Tie-2 (ng/ml)	10.8 (10.0–12.0)	7.7 (5.5–28.3)	0.105
VEGF (pg/ml)	11 (10–110)	120 (72–780)	<0.001
sFlt-1 (ng/ml)	14.0 (4–140)	21.5 (2.5–420)	0.419
vWf (IU/dl)	89 (80–98)	143 (117.3–161)	<0.001
Ratios			
Ang-2/VEGF	100.0 (28.0–136.0)	23.5 (9.2–56.2)	0.009
Ang-2/Ang-1	2.2 (0.6–2.8)	1.4 (0.7–3.1)	0.825
Ang 1/VEGF	50.0 (10–80)	16.5 (4.6–29.9)	0.0058
(Ang-2/Ang-1)×100/VEGF	12.0 (1.7–26.0)	1.4 (0.07–4.6)	0.0002

The study cohort of 56 SCD (26 men, mean (SD) age 33.5 (10) years) patients comprised 36 with HbSC disease and 20 with HbSS disease. Data in the SCD patients were compared with 24 healthy age, sex, and race matched controls (11 men, mean age 34.4 (SD 10) years).
In HbSC and HbSS disease, respective plasma levels of Ang-1 (median 1.7 (IQR 1–11.3) *v* 3.1 (2.0–11.4) ng/ml, p = 0.0571), Tie-2 (7.0 (5.3–28.3) *v* 8.5 (5.7–26.3) ng/ml, p = 0.436), VEGF (115(60–413) *v* 122(103–6625) pg/ml, p = 0.411), or sFlt-1 (13.8 (1.3–295) *v* 48 (11.3–490) ng/ml), p = 0.289), and vWf 133.5 (113.5–155) *v* 151.5 (133.3–168) IU/dl; p = 0.094) did not differ between the groups, although Ang-2 was higher in the SS patients (5.9 (4.4–10) *v* 3.2 (2.0–7.2) ng/ml) p = 0.025).

previous 3 months, had any malignancy, connective tissue disease or vascular disease, diabetes, hypertension, were pregnant, or were on long term medication (such as hydroxyurea). None of the patients had a painful crisis within 2 weeks of the time of ocular examinations and blood sampling. The healthy control group of subjects with normal haemoglobin genotype (AA subjects) were matched for age, sex, and ethnic origin with the PSR patients.

The diagnoses of PSR, NPR, or no retinopathy (NR) were made using slit lamp biomicroscopy and fluorescein angiography.[5] The characteristic confirming feature of the diagnosis of peripheral retinal neovascularisation was intense hyperfluorescence caused by leakage of dye from new blood vessels. Patients with PSR who showed evidence of leakage on fluorescein angiogram (''leaky PSR'') were offered laser treatment with sectoral panretinal photocoagulation (PRP).

A volume of 10 ml of citrated venous blood was obtained for measurement of plasma Ang-1, Ang-2, Tie-2, VEGF, sFlt-1, and vWf. For patients with NR or with NPR, this was the only occasion on which a blood sample was taken. In patients with ''leaky PSR'' a blood sample was repeated at 5–7 (median 6) months after their last laser treatment. Blood samples were taken from the antecubital vein with minimal stasis into Vacuette tubes containing 3.2% sodium citrate and centrifuged at 3000 rpm at 4℃ for 20 minutes. The platelet free plasma was immediately separated and frozen at −70℃. Research indices were measured by ELISA using commercially available reagents and recombinant standards (R&D Systems, Abingdon, UK).

Data are presented as mean (SD) or median (interquartile range (IQR)) and compared by the unpaired *t* test and by the Mann-Whitney U test, or the one way ANOVA and

Table 2 Plasma levels of angiopoietin-1 (Ang-1) angiopoietin-2 (Ang-2), the soluble angiopoietin receptor Tie-2 (Tie-2), vascular endothelial growth factor (VEGF), its soluble receptor Flt-1, and von Willebrand factor (vWf) in patients with different manifestations of sickle eye disease

	NR*	NPR†	PSR‡	p Value
Raw data				
Ang-1 (ng/ml)	2.2 (0.6–10.4)	3.1 (1.2–13.8)	2.0 (1.0–11.3)	0.682
Ang-2 (ng/ml)	6.0 (4.1–9.3)	3.8 (2.0–10.0)	4.7 (2.1–7.4)	0.395
Tie-2 (ng/ml	6.3 (5.0–36.0)	9.0 (6.1–31.5)	7.4 (5.8–22.0)	0.714
VEGF (pg/ml)	108 (61–728)	116 (72–2650)	137 (104–378)	0.748
sFlt-1(ng/ml)	35.0 (3–421)	16.5 (0.3–500)	18.8 (6.1–278)	0.941
vWf (IU/dl)	133 (108–164)	147 (113–166)	143 (122–161)	0.690
Ratios				
Ang-2/VEGF	43.3 (15.5–60.5)	20.5 (2.2–41.5)	18.2 (11.3–59.1)	0.290
Ang-2/Ang-1	2.4 (0.8–4.3)	1.1 (0.6–2.3)	1.6 (0.7–3.1)	0.260
Ang-1/VEGF	14.9 (3.8–27.1)	13.6 (4.1–29.3)	17.2 (4.8–40.6)	0.856
(Ang-2/Ang-1)×100 /VEGF	2.8 (0.2–5.7)	0.8 (0.02–3.5)	1.1 (0.15–4.4)	0.013

The PSR group comprised 22 with HbSC and two with Hb SS (11 men, mean age 35 (SD 11)), NPR comprised nine with HbSC disease and seven with HbSS (seven men aged 34 (10) years), while the NR group was five with HbSC and 11 with Hb SS (eight men, aged 32 (10) years). There were more patients with HbSC in the PSR group (p = 0.008) but the differences in sex and age were not significant (p = 0.936, p = 0.585 respectively).
NR, no retinopathy; NPR, non-proliferative retinopathy; PSR, proliferative retinopathy. *No retinopathy bilaterally; †no retinopathy unilaterally or NPR unilaterally or NPR bilaterally; ‡unilateral or bilateral PSR. Values are median (IQR) except for age, which is expressed as mean (SD). All p values by Mann-Whitney U test except for age which is by the Student's unpaired *t* test.

Table 3 Correlations among plasma angiogenic growth factors, their receptors, and von Willebrand factor in 23 AA subjects and 56 SCD patients

	vWf	VEGF	sFlt-1	Ang-1	Ang-2	Tie-2
23 AA subjects						
VEGF	−0.026, 0.908	–	–	–	–	–
sFlt-1	0.099, 0.624	0.627, 0.001*	–	–	–	–
Ang-1	0.269, 0.215	0.505, 0.014*	0.483, 0.020*	–	–	–
Ang-2	0.209, 0.338	0.369, 0.083	0.244, 0.263	0.245, 0.260	–	–
Tie-2	−0.074, 0.739	0.546, 0.007*	0.526, 0.010*	0.501, 0.015*	–	–
56 SCD patients						
VEGF	0.120, 0.380	–	–	–	–	–
sFlt-1	0.114, 0.401	0.882, <0.001*	–	–	–	–
Ang-1	0.279, 0.037*	0.677, <0.001*	0.610, <0.001*	–	–	–
Ang-2	0.165, 0.225	0.645, <0.001*	0.699, <0.001*	0.591, <0.001*	–	–
Tie-2	0.317, 0.017	0.743, <0.001*	0.766, <0.001*	0.743, <0.001*	0.516, <0.001*	–

Data presented as Spearman correlation coefficient (*r*) followed by p values. *p Values <0.05.
Ang-1: angiopoietin-1, Ang-2: angiopoietin-2, Tie-2: the angiopoietin receptor Tie-2, VEGF, vascular endothelial growth factor, sFlt-1, the VEGF receptor Flt-1; vWf, von Willebrand factor.

Kruskall-Wallis test as appropriate. Correlations were performed using Spearman's rank correlation test. Paired comparisons were tested using the paired Wilcoxon test.

RESULTS

Plasma levels of Ang-1, Tie-2, VEGF, sFlt-1, and vWf did not differ between the groups, although Ang-2 was higher in the SS patients ($p = 0.025$); therefore, data were pooled for further analysis. Plasma levels of Ang-1 and Ang-2 (but not Tie-2 or sFlt-1) were significantly raised in the SCD patients compared to levels in the AA subjects (table 1). As expected,[15 16] VEGF and vWf were higher in SCD. The ratios of Ang-2 to VEGF, Ang-1 to VEGF, and between all three growth factors (that is [Ang-1/Ang-2×100]/VEGF) were lower in the SCD patients than in the controls but there was no statistical difference in the Ang-2/Ang-1 ratio. There were no differences in Ang-1, Ang-2, Tie-2, VEGF, sFlt-1, and vWf levels among the patients (table 2). The ratio [(Ang-1/Ang-2)×100]/VEGF was higher in NR than in the two other groups.

In the AA subjects, the most significant correlations were between Ang-1 and VEGF, Ang-1 and Tie-2, and VEGF and sFlt-1 (table 3). Stronger positive correlations were also apparent between the same molecules in the patients with SCD (table 3). Furthermore, in SCD, Ang-2 was highly correlated with Ang-1, Tie-2 with VEGF, and Ang-1 weakly with vWf. Within each of the SCD subgroups, Ang-1 was significantly correlated with Ang-2, with VEGF and with Tie-2; similarly, Ang-2 was significantly correlated with VEGF and Tie-2, (except in the NPR patients) and VEGF with sFlt-1 (table 4).

Plasma Ang-2 fell but Tie-2 rose significantly in eight patients (one male, mean age 34 (SD 9)) following PRP therapy (table 5), but Ang-1, VEGF, sFlt-1, and vWf levels did not change. Ocular examination showed only partial or incomplete resolution of neovascularisation at the time of the follow up blood sample in all the patients.

DISCUSSION

The current novel findings of elevated plasma Ang-2 in SCD, alongside raised VEGF, are consistent with the concept of increased angiogenic activity in SCD generally. The observations of elevated plasma VEGF[15] (up to ~10-fold) and vWf[16] confirm previous reports. The precise cause for the increased angiogenesis is unclear, but increased systemic tissue hypoxia consequent to generalised subclinical vaso-occlusion may contribute to the elevated plasma Ang-2 and VEGF levels. Marked endothelial damage/dysfunction is associated with SCD[16] and endothelial proliferation as a means of effecting endothelial repair may be a mechanism for attempting to

Table 4 Correlations among angiogenic growth factors

	vWf	VEGF	sFlt-1	Ang-1	Ang-2	Tie-2
16 SCD patients with no retinopathy						
VEGF	−0.331, 0.210	–	–	–	–	–
sFlt-1	−0.015, 0.956	0.743, 0.001*	–	–	–	–
Ang-1	−0.044, 0.872	0.689, 0.003*	0.492, 0.053	–	–	–
Ang-2	0.156, 0.565	0.745, 0.001*	0.881, <0.001*	0.615, 0.011*	–	–
Tie-2	0.156, 0.564	0.459, 0.074	0.742, 0.001*	0.656, 0.006*	0.712, 0.002*	–
16 SCD patients with non-proliferative retinopathy						
VEGF	0.094, 0.730	–	–	–	–	–
sFlt-1	0.076, 0.779	0.881, <0.001*	–	–	–	–
Ang-1	0.320, 0.228	0.756, <0.001*	0.773, <0.001*	–	–	–
Ang-2	0.037, 0.891	0.869, <0.001*	0.761, <0.001*	0.681, 0.004*	–	–
Tie-2	0.413, 0.112	0.701, 0.003*	0.688, 0.003*	0.822, <0.001*	0.475, 0.063	–
24 SCD patients with proliferative retinopathy						
VEGF	0.290, 0.169	–	–	–	–	–
sFlt-1	0.215, 0.313	0.935, <0.001*	–	–	–	–
Ang-1	0.477, 0.018*	0.611, 0.002*	0.470, 0.020*	–	–	–
Ang-2	0.247, 0.245	0.549, 0.005*	0.514, 0.010*	0.589, 0.002*	–	–
Tie-2	0.295, 0.162	0.871, 0.000*	0.831, <0.001*	0.642, 0.001*	0.477, 0.019*	–

Data presented as Spearman correlation coefficient (*r*) followed by p values. *p Values<0.05.
Ang-1: angiopoietin-1, Ang-2: angiopoietin-2, Tie-2: the angiopoietin receptor Tie-2, VEGF: vascular endothelial growth factor, sFlt-1: the VEGF receptor Flt-1 and vWf: von Willebrand factor.

Table 5 The effect of laser treatment (PRP) on plasma levels of angiopoietin-1 (Ang-1) angiopoietin-2 (Ang-2), the soluble angiopoietin receptor Tie-2, VEGF, sFlt-1 and vWf in eight patients with sickle haemoglobin C disease with ''leaky PSR''

	HbSC patients with ''leaky PSR''		
	Baseline (pre-laser)	6 months post-laser	p Value
Raw data			
Ang-1 (ng/ml)	1.2 (0.6–9.5)	0.5 (0.5–1.5)	0.106
Ang-2 (ng/ml)	3.6 (1.5–7.2)	1.1 (1.1–2.0)	0.022
Tie-2 (ng/ml	6.0 (5.5–7.8)	10.0 (7.6–10.6)	0.022
VEGF (pg/ml)	120 (72–138)	115 (50–155)	0.834
sFlt-1(ng/ml)	15 (5–138)	0.1 (0.01–3.6)	0.402
vWf (IU/dl)	133 (113–152)	144 (138–148)	0.673
Ratios			
Ang-2/VEGF	16.1 (12.2–101.1)	20.0 (9.3–23.5)	0.076
Ang-2/Ang-1	3.1 (1.0–4.3)	2.3 (0.77–2.7)	0.035
Ang-1/VEGF	16 (4.8–28.2)	10 (8.8–13.2)	0.151
(Ang-2/Ang-1×100)/VEGF	2.6 (0.5–8.0)	1.7 (0.6–4.6)	0.554

Values are median (IQR) except for age, which is expressed as mean (SD).
All p values by Wilcoxon's paired test.

preserve endothelial homeostasis. As Ang-1 has been shown to have anti-apoptotic effects on endothelial cells,[17 18] we speculate that because of the endothelial damage in SCD, Ang-1 levels are raised in order to provide this support. Our observation of a significant correlation between Ang-1 and vWf in SCD, but not in controls, lends support to this hypothesis.

Destabilisation of growing blood vessels by Ang-2, in the absence of VEGF, leads to vessel regression, whereas such destabilisation in the presence of high VEGF levels facilitates the angiogenic response.[9–11] Thus, the precise balance of VEGF and the angiopoetin/Tie-2 system is important in determining whether or not vessels auto-infarct and regress/atrophy or proliferate and become leaky. We would therefore expect that in PSR patients, retinal vaso-occlusion leads to retinal ischaemia and hypoxia, which induces high levels of VEGF and Ang-2. Conversely, in patients without retinopathy, there might be relatively greater retinal vaso-occlusion possibly also accompanied by greater levels of auto-infarction—leading to less retinal ischaemia and less hypoxia than in PSR patients, and lower levels of VEGF and Ang-2 than in PSR patients. It follows that Ang-2, in the presence of very low levels of VEGF, might induce neovascular regression.

An ''angiogenic index,'' reflecting the ratio of Ang-2 to VEGF and/or the combined angiopoietins to VEGF might be indicators of the presence or development of PSR, with a low index (high Ang-2/high VEGF) indicating the tendency to new vessel proliferation and leakiness, and a high index (mid-high Ang2/low VEGF) indicating tendency to neovascular regression and atrophy. Our observations of lower ratios of the angiopoietins (individually or combined) in SCD compared to AA subjects as well as the trend of lower Ang-2/VEGF from ''no retinopathy'' to PSR is consistent with this. In SCD, the strong intercorrelations among the growth factors, suggest a high degree of coordinated and complementary angiogenic activity and perhaps a role for these factors in abnormal angiogenesis in SCD; however, no clear patterns emerged according to severity of retinopathy.

The role of the angiopoietins and their interaction with VEGF (if any) in retinal neovascularisation is not fully understood. In human retinal tissue, Ang-2 and Tie-2 expression appears to be associated with ischaemic retinal disorders[19] and VEGF expression with abnormal proliferation in SCD.[20] However, the idea that angiogenic activity is further heightened in SCD patients with PSR was not supported by the present findings. The observed lack of difference in any molecule among the SCD patients may be attributable to their existing elevated levels that may obscure any further increase in their levels in different eye conditions.

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Authors' affiliations

J S Mohan, A D Blann, G Y H Lip, Haemostasis, Thrombosis and Vascular Biology Unit, University Department of Medicine, The Birmingham and Midland Eye Centre, City Hospital, Birmingham B18 7QH, UK
J S Mohan, D Bareford, Department of Haematology, The Birmingham and Midland Eye Centre, City Hospital, Birmingham B18 7QH, UK
P L Lip, Department of Ophthalmology, The Birmingham and Midland Eye Centre, City Hospital, Birmingham B18 7QH, UK

Correspondence to: Professor G Y H Lip, University Department of Medicine, City Hospital, Birmingham B18 7QH, UK; g.y.h.lip@bham.ac.uk

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