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 Barendorf, G Y H Lip

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.

Changes in both plasma and intraocular VEGF levels have been related to laser treatment. 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.

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. 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. Ang-2, conversely, leads to destabilisation of vessels and dissociation of pericytes, and is upregulated by hypoxia and angiogenic cytokines, including VEGF and in pathological angiogenesis associated with tumours and choroidal neovascularisation associated with age related macular degeneration. Thus, the precise balance of VEGF and the angiopoietin/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 but any role in associated eye disease is unclear. We therefore hypothesised abnormal Ang-1, Ang-2, sFlt-1, and Tie-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 (HbS 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 transfusion, had blood transfusion within the 1 month prior to study, or had an estimated creatinine clearance of less than 60 ml/min.

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
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). None of the patients had a painful crisis 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°C for 20 minutes. The platelet free plasma was immediately separated and frozen at −70°C. 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 by the Student's unpaired t test.

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)

<table>
<thead>
<tr>
<th></th>
<th>AA subjects</th>
<th>SCD patients</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 23) (IQR)</td>
<td>(n = 56) (IQR)</td>
<td></td>
</tr>
<tr>
<td>Ang-1 (ng/ml)</td>
<td>0.5 (0.5–2.5)</td>
<td>2.2 (1.0–11.4)</td>
<td>0.0004</td>
</tr>
<tr>
<td>Ang-2 (ng/ml)</td>
<td>1.3 (1.0–2.0)</td>
<td>5.1 (2.3–7.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Tie-2 (ng/ml)</td>
<td>10.8 (10.0–12.0)</td>
<td>7.7 (5.5–28.3)</td>
<td>0.105</td>
</tr>
<tr>
<td>VEGF (gg/ml)</td>
<td>11 (10–110)</td>
<td>120 (72–780)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>sfFlt-1 (ng/ml)</td>
<td>14.0 (4–140)</td>
<td>21.5 (2.5–420)</td>
<td>0.419</td>
</tr>
<tr>
<td>vWF (IU/dl)</td>
<td>89 (80–98)</td>
<td>143 (117.3–161)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

The diagnoses of PSR, NPR, or no retinopathy (NR) were made using slit lamp biomicroscopy and fluorescein angiography. 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°C for 20 minutes. The platelet free plasma was immediately separated and frozen at −70°C. 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 by the Student's unpaired t test.

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

<table>
<thead>
<tr>
<th></th>
<th>NR*</th>
<th>NPR†</th>
<th>PSR‡</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ang-1 (ng/ml)</td>
<td>2.2 (0.6–10.4)</td>
<td>3.1 (1.2–13.8)</td>
<td>2.0 (1.0–11.3)</td>
<td>0.682</td>
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<tr>
<td>Ang-2 (ng/ml)</td>
<td>6.0 (4.1–9.3)</td>
<td>3.8 (2.0–10.0)</td>
<td>4.7 (2.1–7.4)</td>
<td>0.395</td>
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<tr>
<td>Tie-2 (ng/ml)</td>
<td>6.3 (5.0–36.0)</td>
<td>9.0 (6.1–31.5)</td>
<td>7.4 (5.9–22.0)</td>
<td>0.714</td>
</tr>
<tr>
<td>VEGF (gg/ml)</td>
<td>108 (61–728)</td>
<td>116 (72–2650)</td>
<td>137 (104–378)</td>
<td>0.748</td>
</tr>
<tr>
<td>sfFlt-1 (ng/ml)</td>
<td>35.0 (3–421)</td>
<td>16.5 (0.3–500)</td>
<td>18.8 (6.1–278)</td>
<td>0.941</td>
</tr>
<tr>
<td>vWF (IU/dl)</td>
<td>133 (108–164)</td>
<td>147 (113–166)</td>
<td>143 (122–161)</td>
<td>0.690</td>
</tr>
</tbody>
</table>

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 Hb SS disease. The NR group comprised five with HbSC disease and seven with Hb SS disease (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.
Kruskall-Wallis test as appropriate. Correlations were performed using Spearman’s rank correlation test. Paired comparisons were used 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, 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]/VEGF) were increased in SCD generally. The observations of elevated plasma VEGF (up to 10-fold) and vWF in SCD patients (non-proliferative patients) 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 and endothelial proliferation as a means of effecting endothelial repair may be a mechanism for attempting to

<table>
<thead>
<tr>
<th>23 AA subjects</th>
<th>vWF</th>
<th>VEGF</th>
<th>sFlt-1</th>
<th>Ang-1</th>
<th>Ang-2</th>
<th>Tie-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>-0.026, 0.908</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>sFlt-1</td>
<td>0.099, 0.624</td>
<td>0.627, 0.001*</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Ang-1</td>
<td>0.269, 0.145</td>
<td>0.505, 0.014*</td>
<td>0.483, 0.020*</td>
<td>-</td>
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<tr>
<td>Ang-2</td>
<td>0.209, 0.0338</td>
<td>0.369, 0.083</td>
<td>0.244, 0.263</td>
<td>0.245, 0.260</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Tie-2</td>
<td>-0.074, 0.739</td>
<td>0.546, 0.007*</td>
<td>0.526, 0.010*</td>
<td>0.501, 0.015*</td>
<td>-</td>
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</table>

<table>
<thead>
<tr>
<th>56 SCD patients</th>
<th>vWF</th>
<th>VEGF</th>
<th>sFlt-1</th>
<th>Ang-1</th>
<th>Ang-2</th>
<th>Tie-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>0.120, 0.380</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>sFlt-1</td>
<td>0.114, 0.401</td>
<td>0.882, &lt;0.001*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Ang-1</td>
<td>0.279, 0.037*</td>
<td>0.677, &lt;0.001*</td>
<td>0.610, &lt;0.001*</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ang-2</td>
<td>0.165, 0.225</td>
<td>0.645, &lt;0.001*</td>
<td>0.699, &lt;0.001*</td>
<td>0.591, &lt;0.001*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tie-2</td>
<td>0.317, 0.017</td>
<td>0.743, &lt;0.001*</td>
<td>0.766, &lt;0.001*</td>
<td>0.743, &lt;0.001*</td>
<td>0.516, &lt;0.001*</td>
<td>-</td>
</tr>
</tbody>
</table>

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.

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 (up to 10-fold) and vWF in SCD patients 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 and endothelial proliferation as a means of effecting endothelial repair may be a mechanism for attempting to
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.19 20 Thus, the precise balance of VEGF and the angiopoietin/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-oclusion 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-oclusion 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 disorders19 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.

ACKNOWLEDGEMENTS

We thank the staff of the Sickle Cell and Thalassemia Centre, City Hospital, for their support of this research. We gratefully acknowledge the funding of the Sandwell and West Birmingham Hospitals NHS Trust Research and Development programme for the Haemostasis Thrombosis and Vascular Biology Unit.

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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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REFERENCES


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”

<table>
<thead>
<tr>
<th>Raw data</th>
<th>Baseline (pre-laser)</th>
<th>6 months post-laser</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>vWF (IU/dl)</td>
<td>133 (113–152)</td>
<td>144 (138–148)</td>
<td>0.673</td>
</tr>
<tr>
<td>sFlt-1 (ng/ml)</td>
<td>15 (5–138)</td>
<td>0.1 (0.01–3.6)</td>
<td>0.402</td>
</tr>
<tr>
<td>VEGF (pg/ml)</td>
<td>120 (72–138)</td>
<td>115 (50–155)</td>
<td>0.834</td>
</tr>
<tr>
<td>Tie-2 (ng/ml)</td>
<td>6.0 (5.5–7.8)</td>
<td>10.0 (7.6–10.6)</td>
<td>0.022</td>
</tr>
<tr>
<td>Ang-2 (ng/ml)</td>
<td>3.6 (1.5–7.2)</td>
<td>1.1 (1.1–2.0)</td>
<td>0.022</td>
</tr>
<tr>
<td>Ang-1 (ng/ml)</td>
<td>1.2 (0.6–9.5)</td>
<td>0.5 (0.5–1.5)</td>
<td>0.106</td>
</tr>
<tr>
<td>Ang-2/VeGF</td>
<td>16.1 (12.2–101.1)</td>
<td>20.0 (9.3–22.5)</td>
<td>0.076</td>
</tr>
<tr>
<td>Ang-2/Ang-1</td>
<td>3.1 (1.0–4.3)</td>
<td>2.3 (0.77–2.7)</td>
<td>0.035</td>
</tr>
<tr>
<td>Ang-1/VEGF</td>
<td>16.4 (4.8–28.2)</td>
<td>10.8 (8.13–12.2)</td>
<td>0.151</td>
</tr>
<tr>
<td>(Ang-2/Ang-1 x 100)/VEGF</td>
<td>2.6 (0.5–8.0)</td>
<td>1.7 (0.6–4.6)</td>
<td>0.554</td>
</tr>
</tbody>
</table>

Values are median (IQR) except for age, which is expressed as mean (SD).

All p values by Wilcoxon’s paired test.

Table 5
Angiopoietin/Tie-2 in proliferative sickle retinopathy


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