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

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 PSR (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 (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 transfusion, had blood transfusion 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
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 Vacutette 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
Angiopoietin/Tie-2 in proliferative sickle retinopathy

**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 follow up blood sample in all the patients.

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).

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

<table>
<thead>
<tr>
<th></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>23 AA subjects</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<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>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ang-1</td>
<td>0.269, 0.014</td>
<td>0.350, 0.001*</td>
<td>0.483, 0.020*</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ang-2</td>
<td>0.209, 0.083</td>
<td>0.244, 0.263</td>
<td>0.245, 0.260</td>
<td>0.252, 0.010*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tie-2</td>
<td>-0.074, 0.739</td>
<td>0.546, 0.000*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>56 SCD patients</td>
<td></td>
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<td></td>
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<tr>
<td>VEGF</td>
<td>0.120, 0.380</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>
</tr>
<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>0.991, &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.

**Table 4** Correlations among angiogenic growth factors

<table>
<thead>
<tr>
<th></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>16 SCD patients with no neovascularisation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td>-0.331, 0.210</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>sFlt-1</td>
<td>-0.015, 0.956</td>
<td>0.743, 0.001*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ang-1</td>
<td>-0.044, 0.872</td>
<td>0.689, 0.003*</td>
<td>0.492, 0.053</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ang-2</td>
<td>0.156, 0.565</td>
<td>0.745, 0.001*</td>
<td>0.881, &lt;0.001*</td>
<td>0.615, 0.011*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tie-2</td>
<td>0.156, 0.564</td>
<td>0.459, 0.074</td>
<td>0.742, 0.001*</td>
<td>0.656, 0.005*</td>
<td>0.712, 0.002*</td>
<td>-</td>
</tr>
<tr>
<td>16 SCD patients with non-proliferative neovascularisation</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>VEGF</td>
<td>0.094, 0.730</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>sFlt-1</td>
<td>0.076, 0.779</td>
<td>0.881, &lt;0.001*</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>Ang-1</td>
<td>0.320, 0.226</td>
<td>0.756, &lt;0.001*</td>
<td>0.773, &lt;0.001*</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>Ang-2</td>
<td>0.037, 0.891</td>
<td>0.869, &lt;0.001*</td>
<td>0.761, &lt;0.001*</td>
<td>0.681, 0.004*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tie-2</td>
<td>0.413, 0.112</td>
<td>0.701, 0.003*</td>
<td>0.688, 0.003*</td>
<td>0.822, &lt;0.001*</td>
<td>0.475, 0.063</td>
<td>-</td>
</tr>
<tr>
<td>24 SCD patients with proliferative neovascularisation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td>0.290, 0.169</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>sFlt-1</td>
<td>0.215, 0.313</td>
<td>0.935, &lt;0.001*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ang-1</td>
<td>0.477, 0.018*</td>
<td>0.611, 0.002*</td>
<td>0.470, 0.020*</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ang-2</td>
<td>0.247, 0.245</td>
<td>0.549, 0.005*</td>
<td>0.514, 0.010*</td>
<td>0.589, 0.002*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tie-2</td>
<td>0.295, 0.162</td>
<td>0.871, 0.000*</td>
<td>0.831, &lt;0.001*</td>
<td>0.642, 0.001*</td>
<td>0.477, 0.019*</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.
preserve endothelial homeostasis. As Ang-1 has been shown to have anti-apoptotic effects on endothelial cells, 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. Thus, the precise balance of VEGF and the angiopoietin-Tie-2 system is important in determining whether or not vessels auto-infarct and regress/arrotphy 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 and VEGF expression with abnormal proliferation in SCD. 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

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