Sickle cell disease has the highest incidence for a population at risk of any genetically derived disease. Three in every 1000 African Americans born have sickle cell anemia (SS disease). Sickle cell disease is caused by a point mutation in the β globin gene. Impairment of blood flow accounts for nearly all clinical manifestations of the sickling syndrome. Vasoo-cclusion occurs in most organs, often causing morbidity, including splenomegaly, liver necrosis, painful crisis (largely narrow infarcts), acute chest syndrome, and retinopathy.

The initial retinal occlusions occur in periphery and have been observed in children with SS disease less than 2 years of age. The peripheral occlusions in children are primarily in capillaries but, with age, the occlusions occur in major blood vessels as well, and the peripheral retina becomes avascular. At the sites of occlusion, hairpin loops and arteriovenous anastomoses may form, resulting in abnormal blood flow. Pre-retinal neovascularisation, called sea fans, also forms adjacent to the preretinal neovascular in subjects with proliferative retinopathy. This was not the case with VCAM-1 immunoreactivity, which was highest in intraretinal vessels adjacent to the sea fan when the sea fan was still "in stitu nascendi." Fully formed, "older" sea fans had the highest levels of VCAM-1. The increase in adhesion molecule immunoreactivity was paralleled by an increase in intraretinal PMNs. The number of intraretinal PMNs increased with progression of the disease and the numbers surpassed those in control subjects by threefold. In the sea fan with the greatest VCAM-1 immunoreactivity, there were 20 times more PMNs were observed than in the rest of the retina in the same subject.

Conclusion: These data suggest that adhesion molecule mediated leucocyte adhesion might play an important part in the vaso-occlusive phase of sickle cell retinopathy and in autoinfarction of sea fan formations.

Aims: The expression of the adhesion molecules ICAM-1, VCAM-1, and P-selectin, and the distribution and number of polymorphonuclear leukocytes (PMNs) were investigated in sickle cell retinopathy (SCR) and compared to the normal retina.

Methods: Postmortem ocular tissue was obtained from five subjects (16, 21, 28, 40, and 41 years of age) with sickle haemoglobinopathies and from one control subject. Tissue was cryopreserved, and streptavidin peroxidase immunohistochemistry was performed with antibodies against ICAM-1, VCAM-1, and P-selectin. Immunohistochemical reaction product was scored, and PMN numbers were counted in sections stained with non-specific esterase.

Results: Increased ICAM-1, VCAM-1, and P-selectin immunoreactivities were observed in sickle cell subjects compared to the control subject. The highest ICAM and P-selectin immunoreactivity was associated with intraretinal vessels adjacent to the preretinal neovascular formation in subjects with proliferative retinopathy. This was not the case with VCAM-1 immunoreactivity, which was highest in intraretinal vessels adjacent to the sea fan when the sea fan was still “in stitu nascendi.” Fully formed, “older” sea fans had the highest levels of VCAM-1. The increase in adhesion molecule immunoreactivity was paralleled by an increase in intraretinal PMNs. The number of intraretinal PMNs increased with progression of the disease and the numbers surpassed those in control subjects by threefold. In the sea fan with the greatest VCAM-1 immunoreactivity, there were 20 times more PMNs were observed than in the rest of the retina in the same subject.

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Neutrophils and leucocyte adhesion molecules in sickle cell retinopathy

M Kunz Mathews, D S McLeod, C Merges, J Cao, G A Lutty

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Conclusion: These data suggest that adhesion molecule mediated leucocyte adhesion might play an important part in the vaso-occlusive phase of sickle cell retinopathy and in autoinfarction of sea fan formations.

Abbreviations: ICAM-1, intercellular adhesion molecule 1; IL-1α, interleukin α (IL-1α); NSE, non-specific esterase; PMN, polymorphonuclear leukocytes; SCR, sickle cell retinopathy; sRBCs, sickle erythrocytes; TNFα, tumour necrosis factor α; VCAM-1, vascular adhesion molecule 1; WBC, white blood cells; vWF, von Willebrand factor
potent neutrophil chemokine IL-8 is also elevated in sera of sickle cell subjects during crisis.25

The purpose of the current study was to investigate the prevalence and localisation of the leucocyte adhesion molecules ICAM-1, VCAM-1, and P-selectin in sickle cell retina and in preretinal neovascular formations. The numbers of PMNs in retina and sea fan formations were also determined. The study demonstrates that all three adhesion molecules are upregulated and the number of PMNs elevated in sickle cell retina and further elevated in sea fans. The increased presence of PMNs and their adhesion molecules suggests that these leucocytes may participate in retinal vascular vaso-occlusive processes and autoinfarction of preretinal neovascularisation.

**MATERIALS AND METHODS**

Human postmortem eyes were obtained from Alabama Eye Bank (Mobile, AL, USA), Mid-America Eye and Tissue Bank (St Louis, MO, USA), and Medical Eye Bank of Maryland (Baltimore, MD, USA) and used with the approval of the Johns Hopkins Hospital joint committee on clinical investigations. One preretinal membrane (case 1) was obtained from a vitrectomy sample taken at the Johns Hopkins Hospital. In addition, cadaver eyes from five subjects with a history of sickle cell disease and one normal subject were examined. The normal subject was used previously in a study of leucocyte adhesion molecules and PMNs in diabetic retinopathy and was found to be representative of the six control, non-diabetic subjects used in that previous study.26

In the sickle cell group, the average age was 29 years (range 16–41 years), the average postmortem (or postoperative in case 1) time was 11 hours (range 2–31 hours), and the average death to enucleation time 3.75 hours (range 2–5.5 hours). In total, nine samples were examined from five sickle cell subjects (Table 1). Five of the eight sickle cell samples were obtained from the superior region of retina, and two samples from the inferior region of retina. All samples were serial sectioned, obtaining sections from three different preretinal neovascular formations (sea fans) and their surrounding regions of retina, and from four areas of sickle cell retina without preretinal neovascularisation. Thus, we could examine normal versus sickle cell retina, proliferative versus non-proliferative areas of sickle cell retina, and superior versus inferior region of sickle cell retina since retinopathy is said to be more prevalent in superior retina.27

Immunohistochemistry was performed on serial 8 μm sections of cryopreserved tissue as previously described.28 In brief, the sections (two sections per slide) were incubated with primary antibodies for 20 hours at 4°C with the antibodies applied in the following order to serial sections: rabbit anti-von Willebrand factor (vWf, Dako Co, Carpintera, CA, USA) at 0.57 μg/ml; mouse anti-VCAM-1 (Genosys Inc, Woodlands, TX, USA) at 0.002 μg/ml; mouse anti-ICAM-1 (kindly provided by Robert Rothlein, Boehringer Ingelheim Inc, Ridgefield, CT, USA) at 2.6 μg/ml; mouse anti-P-selectin (Cytel Inc, San Diego, CA, USA) at 12.6 μg/ml; and rat anti-perlecan diluted 1:5000 (graciously provided by Alex Ljubimov, PhD, Cedar Sinai Medical Center, Los Angeles, CA, USA). Each primary antibody was applied to duplicate slides in each experiment. Second step antibodies were biotinylated and streptavidin peroxidase was used as the third step (Kirkegaard and Perry Laboratories Inc, Gaithersburg, MD, USA). Peroxidase was developed with aminomethylcarbazol yielding a red

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SC = heterozygote for haemoglobin S and C; SS = homozygote for haemoglobin S or sickle cell anaemia; AA, normal β globin; BF = black female; BM = black male; PMT = postmortem time; DET = death to enucleation time.
reaction product. One of the two sections on each slide was counterstained with haematoxylin. Control sections were incubated with non-immune IgG (Jackson ImmunoResearch, Westgrove, PA, USA) from the same species and at the same protein concentration as the primary antibody. Sections from the control subject and at least three sickle cell specimens were included in each immunohistochemical experiment.

Qualitative analysis was performed by three masked investigators on a Zeiss light microscope with a 16× objective. The relative amount of immunoreactivity was evaluated using the seven point grading system of Page et al that we have previously reported for adhesion molecules. The average (SD) grades for all three investigators were then determined.

A slide in each series was stained with non-specific esterase (NSE) that stains granulocytes, as reported previously. PMN numbers were counted manually on all sections stained with NSE by two investigators and the average PMN numbers (SD)/mm² of retina were then determined.

As a measure of quality control for our scoring system and a more accurate semiquantitative measure of immunoreactivity levels, we performed densitometric analysis of ICAM-1, VCAM-1, and P-selectin immunoreactivities in cases 6 and 4 as follows. We captured images of the same fields in serial sections on the non-counterstained sections using our image analysis system consisting of a Zeiss Photomicroscope II, a Hamamatsu Video Camera (model No C2400–77, Hamamatsu City, Japan), and the NIH-Image 1.47 software on a Macintosh IIfci computer (Apple, Cupertino, CA, USA). On each section examined, we measured relative grey scale values of all vessels in retina and in the sea fans as we have described previously.

Statistical evaluation of the immunohistochemical data (scores from three observers of triplicate slides from each subject incubated with each antibody) and PMN numbers was performed using the Student’s t tests. p Values equal to or less than 0.05 were considered significant.

RESULTS

A brief description of the subjects used in this study is presented in Table 1. In the sickle cell group, two patients had sickle haemoglobin type SS (sickle cell anaemia) and three subjects had SC haemoglobin (SC disease) (Table 1). Three subjects had proliferative retinopathy while two had non-proliferative retinopathy. The sea fans in the specimens had different morphological features (Fig 1). One sea fan was composed primarily of large dilated lumens (Fig 1A), one was composed primarily of viable capillary-like lumens (Fig 1B), and one was partially infarcted (Fig 1C), as demonstrated by immunohistochemical localisation of perilipin, which we have previously demonstrated is present only in viable blood vessels.

VCAM-1 immunoreactivity was generally elevated in proliferative sickle cell specimens when compared to control and was associated mostly with blood vessels in retina (Fig 2). The greatest difference in VCAM-1 immunoreactivity between control and sickle cell retina was noted for case 4 (p<0.0001) (Fig 3A). VCAM-1 immunoreactivity was highest in sea fans (Fig 4). Comparing scores in retina and in the sea fans of each subject, we found a significant increase in VCAM-1 immunoreactivity in sea fans from case 4 (sea fan in Fig 1A p<0.0001; sea fan in Fig 1B p=0.017) (Fig 3A). The vitrectomy specimen (case 1) and cases 2 and 3 also had elevated VCAM-1 immunoreactivity when compared with control, but the difference was not statistically significant. When superior and inferior regions of retina were compared, we found a statistically significant increase in VCAM-1 immunoreactivity for case 4 between superior and inferior (proliferative sickle cell retinopathy (SCR)) (p<0.0001), but not for case 5 (non-proliferative SCR) whose VCAM-1 scores were comparable to control in both areas of retina (Fig 5A).

The densitometry results confirmed our scoring data (results not shown). Relative intraretinal grey scale values in the sickle cell subject examined (case 4) were significantly higher than in control (case 6, p<0.0001). The relative grey scale values in both sea fans were also higher than the intraretinal grey scale values from case 4 (sea fans in Fig 1A and B), but only the value for the sea fan in Figure 1A was statistically significant (p<0.0001).

P-selectin immunoreactivity was not observed in the control subject. In all sickle cell subjects, we found a statistically significant elevation of P-selectin immunoreactivity when compared to control. The greatest difference in immunoreactivity between control and sickle cell retina was again noted for case 4 (p<0.0001). P-selectin immunoreactivity was also elevated in preretinal formations. However, there was no statistically significant difference between sickle cell retina and sea fan in the same subject. There was significantly more P-selectin immunoreactivity in superior than inferior regions for the subject with proliferative SCR (case 4, p=0.04) as well as for the subject with non-proliferative SCR (case 5, p=0.05).

ICAM-1 immunoreactivity was generally elevated in sickle cell specimens in all areas of retina and in sea fans, whether the subject had proliferative or non-proliferative retinopathy. Unlike VCAM-1, the differences between intraretinal and preretinal ICAM-1 scores were very small. In two samples (case 4, sea fan in Fig 1B, and case 2), the preretinal ICAM-1 immunoreactivity was even lower than in retina, but it was still considerably elevated when compared to control. In the vitrectomy specimen (case 1), ICAM-1 immunoreactivity was comparable to control. ICAM-1 scores in the inferior region of
We observed an increase in neutrophil numbers per mm² of retina in sickle cell retina in general, when compared with control, which was statistically significant for cases 3, 4 (area B), and 5 (p = 0.0002, = 0.007, and <0.02 respectively). The largest number of intraretinal neutrophils was seen in case 3. In the sea fans from case 4 (Figs 1A, B) and case 2 (Fig 1C), neutrophil numbers were increased 305-fold, 81-fold, and 67-fold respectively when compared with intraretinal control PMN numbers. In the steroid treated case 1, no increase in neutrophil numbers was noted. When superior and inferior areas of retina were compared, there were fewer neutrophils in inferior retina in both cases, which was statistically significant for case 5 (p<0.02), but not for case 4.

In summary, VCAM-1 was elevated in all sickle cell subjects, but the increase in reaction product was only significant when sea fans were compared with localization in control subject's retina (Fig 7A). ICAM-1 immunoreactivity was also elevated in sickle cell group compared to control subject, but the difference was only significant for areas of retina sectioned and not for sea fans (Fig 7B). P-selectin immunoreactivity was significantly elevated in all areas of retina and in sea fans compared to control subjects (Fig 7C). Neutrophil numbers were significantly elevated in all areas of retina and in sea fans compared to control subjects (Fig 6) (p <0.007 and <0.0001 respectively). The number of PMNs in sea fan formations was significantly greater than the number in sickle cell retina (p <0.0001).

**DISCUSSION**

Vaso-occlusion is the initial event in sickle cell retinopathy. Although it is intuitive that sickle RBCs would be the initiating cell type in the vaso-occlusive process, the current study suggests that neutrophils may contribute as well. This seems likely since sickle cell disease subjects have high circulating cytokine levels, high white cell counts, and frequent infections. In the current study, the number of PMNs was elevated in the sickle cell retina and in sea fan formations. Sickle PMNs are more adherent to fibronectin than normal PMNs, and sickle cell subjects have more activated PMNs than non-sickle cell subjects. RBCs adhere to PMNs in sickle cell disease and may initiate the PMN's respiratory burst. We have observed PMNs in RBC cell plugs at sites of occlusions in sickle cell retinopathy.

Not only were increased numbers of PMNs observed in the current study, but the adhesion molecules responsible for PMN rolling and firm adherence, P-selectin and ICAM-1 respectively, were elevated as well in sickle cell retina and in sea fan formations. ICAM-1 production can be stimulated by VEGF. Cao et al observed greatly elevated VEGF in the same sea fan formations studied in the current paper. ICAM-1 expression is also shear stress regulated, and shear stress may be elevated in sickle cell subjects. In a previous study of adhesion molecules in diabetic retina, ICAM-1 was elevated in diabetic retina but P-selectin was rarely observed, yet in the sickle cell subjects studied, both P-selectin and ICAM-1 were significantly elevated in retina.

Of the adhesion molecules studied and found to be elevated in this study, VCAM-1 may be the most significant for progression of vaso-occlusive processes. In binding its counter-receptor VLA-4 (α4β1), it can initiate the adherence of B and T lymphocytes, and monocytes. Several groups have recently demonstrated that some reticulocytes from sickle cell subjects have VLA-4 on their surface as well. These cells could be stress reticulocytes, which are prematurely released from marrow because of the rapid destruction of circulating RBCs in sickle cell subjects. VCAM-1 has been reported to mediate sickle reticulocyte binding to human endothelium in vitro via VLA-4. Setty and Stuart have found that VCAM-1/VLA-4 interaction is responsible for the in vitro adherence to both macrovascular and retinal microvascular endothelial
cells. Elevated serum levels of soluble VCAM-1 have been observed in sickle cell subjects with acute chest syndrome, another vaso-occlusion initiated event in the sickle cell subject, suggesting that VCAM-1 may be overexpressed in other organs during pathological events.

Using a rat model, we have observed adherence of reticulocytes via VLA-4 after administration of TNFα to the rats. Blocking VLA-4 with a cyclic peptide or monoclonal antibody has been shown to prevent sickle RBC adherence in our rat model of sickle cell mediated retinal vascular occlusion. However, in that model, fibronectin appeared to be the counter-receptor for VLA-4, not VCAM-1. Kaul's work in mesoacuum and mesoappendix suggest that adherence of lighter density cells initiates the vaso-occlusive process and dense cells (ISC) may be trapped upstream at sites of reticuloocyte adherence. Adhesion of reticulocytes to endothelial cells would also cause leucostasis owing to changes in flow patterns, which increases the opportunity for leucocytes to firmly adhere to their endothelial adhesion molecules like ICAM-1.

Cytokines like TNFα and IL-1α upregulate leucocyte adhesion molecule expression by endothelial cells and activated PMNs. Both TNFα and IL-1α are elevated in steady state sickle cell subjects, perhaps because of a low level of inflammation caused by abnormal adherence of sRBCs to endothelial cells in the microvasculature. The potent neutrophil chemo-kine IL-8 is also elevated in sickle cell subjects during crisis. IL-6 is also elevated in the plasma of sickle cell subjects and it stimulates adherence of sickle PMNs and RBCs to fibronectin. Alternatively, it may be the sickle erythrocytes themselves that cause the upregulation of the leucocyte adhesion molecules observed in this study. Shiu et al recently demonstrated in vitro, using endothelial cells in flow chambers, that ICAM-1 and VCAM-1 gene expression increased in endothelial cells after perfusion with sickle RBCs.

The localisation and relative levels of adhesion molecules were assessed in several locations in the sickle cell retina. Inferior retina was compared with superior because retinopathy occurs less in inferior retina. No significant difference or trend in adhesion molecule levels were observed between superior and inferior retina in the two cases used for that analysis. We also did not see a difference in adhesion molecule localisation between SS and SC subjects (Table 1), although case 4, an SS subject, had the highest levels of all adhesion molecules. We compared localisation and relative levels of reaction product between perfused areas and the border of perfused and non-perfused retina, as determined by vWF and perlecan localisation in serial sections. The border area could potentially be hypoxic because the continuum of vaso-occlusive events in sickle cell retina occurs in the border area most often. Furthermore, ICAM-1 can be induced by hypoxia. The border area had the highest level of ICAM-1 in almost all specimens, but the difference between border and perfused retina was not significant. For VCAM-1, the perfused area tended to have greater levels than the border areas but, again, the difference was not significant. Sea fan formations had the highest VCAM-1 levels of all areas. The sea fan from case 1, the vitrectomy specimen, had the lowest ICAM-1 and VCAM-1 scores of any of the preretinal membranes. This may be related to the steroid treatment the patient received before surgery or, alternatively, could be related to this specimen having the shortest postmortem (postoperative) time of any specimen.

Sea fan formations also had the greatest number of PMNs. This suggests that PMNs may contribute to the autoinfarction of these neovascular formations that has previously been reported. Poor flow properties of these pathological vasculatures could also contribute to the retention of PMNs in sea fans. None of the eyes used in this study was perfused before preparation for freezing, so we assume that the numbers of PMNs in the sections represent those present in retina at the death of the subject.

In summary, the current study has demonstrated that leucocyte adhesion molecules are elevated in sickle cell retina. VCAM-1 was elevated in sickle cell retina and especially in preretinal neovascular formations. Expression of VCAM-1 in sickle cell retina can modulate the adherence of B and T lymphocytes, monocytes, and sickle reticulocytes. ICAM-1 and P-selectin were elevated in sickle cell retina and sea fan formations, which can result in increased adhesion of PMNs to vascular endothelium. The number of PMNs in these sickle cell retinas was greatly elevated. If PMNs do not initiate the
vaso-occlusions, they may contribute to finalising the occlusive event. They can destroy endothelial cells with their oxidative burst, exposing basement membrane, and allowing platelet adhesion. We have observed PMNs in platelet fibrin thrombi at sites of vaso-occlusions in sickle cell subjects.

Increase in PMNs in the sickle cell retina is one of the many pleiotropic effects of this disease caused by a point mutation in the haemoglobin gene. The pleiotropic effects are further modulated by the epistatic effects like upregulation of the adhesion molecule expression.

The positive effects of hydroxyurea treatment, one of the few therapies available to
most sickle cell subjects, may in part be due to inactivation of PMNs and reduction in production of endothelial cell VCAM-1, although soluble VCAM-1 levels were not significantly reduced during hydroxyurea therapy in a recent study. In conclusion, adhesion molecule mediated leucocyte adhesion might have an important role in retinal vaso-occlusive processes and autoinflammation of sea fans.

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References

14 Blei F, Fancher T, Guarnier L. Elevated levels of circulating molecule of potential endothelial cell origins in sickle cell disease. Blood 1994;84(suppl 1):409a.
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