Microvascular abnormalities in sickle cell disease: a computer-assisted intravital microscopy study


The conjunctival microcirculation of 18 homozygous sickle cell disease (SCD) patients during steady-state, painful crisis, and postcrisis conditions was recorded on high-resolution videotapes using intravital microscopy. Selected videotape sequences were subsequently coded, frame-captured, studied, and blindly analyzed using computer-assisted image analysis protocols. At steady-state (baseline), all SCD patients exhibited some of the following morphometric abnormalities: abnormal vessel diameter, comma signs, blood sludging, boxcar blood flow phenomenon, distended vessels, damaged vessels, hemosiderin deposits, vessel tortuosity, and microaneurysms. There was a decrease in vascularity (diminished presence of conjunctival vessels) in SCD patients compared with non-SCD controls, giving the bulbar conjunctiva a “blanched” avascular appearance in most but not all SCD patients during steady-state. Averaged steady-state red cell velocity in SCD patients was slower than in non-SCD controls. During painful crisis, a further decrease in vascularity (caused by flow stoppage in small vessels) and a 36.7% ± 5.2% decrease in large vessel (mostly venular) diameter resulted. In addition, the conjunctival red cell velocities either slowed significantly (6.6% ± 13.1%; P < .01) or were reduced to a trickle (unmeasurable) during crisis. The microvascular changes observed during crisis were transient and reverted to steady-state baseline after resolution of crisis. When combined, intravital microscopy and computer-assisted image analysis (computer-assisted intravital microscopy) represent the availability of a noninvasive tool to quantify microvascular abnormalities in vascular diseases, including sickle cell disease. The ability to identify and relocate the same conjunctival vessels for longitudinal studies uniquely underscores the applicability of this quantitative real-time technology.

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Introduction

Sickle cell disease (SCD) is a compendium of genetic diseases that primarily includes homozygous sickle cell anemia (HbSS), compound heterozygous combinations of HbS and β thalassemia (HbS-thal), and heterozygous (HbS-HbC) disease (HbSC). A single amino acid substitution (Glu to Val) in the β-globin chain of hemoglobin in SCD patients leads to myriad clinical effects and complications. With much improved treatment for infections and the availability of blood transfusion products for hypoplastic crisis, the life expectancy of patients with HbSS has improved considerably since 1960. Nonetheless, SCD patients have significantly decreased survival rates when compared with age-, sex-, and ethnic-matched control groups. Vascular pathology (vasculopathy) underlies most of the complications and accounts for much of the morbidity and mortality. However, real-time studies characterizing the in vivo microvascular abnormalities of SCD patients have rarely been conducted.

Sludging of blood and the presence of comma signs in the vessels of the bulbar conjunctiva in SCD patients were first noted decades ago by Knisely et al and Paton, respectively. Blood sludging and comma signs are caused by stagnation of blood flow in small conjunctival vessels resulting in extremely slow or no blood movement; sludging can be seen when midsized to large-sized vessels are compacted with red cells ("S" in Figures 1B-D and 2), while comma signs are seen as short columns of stationary red cells in small obstructed vessels ("CS1" and "CS2" in Figure 2, for the 2 unique shapes of comma signs observed). When blood flow in the small conjunctival vessels (mostly in arterioles and sometimes small venules) is sluggish or intermittent, the boxcar blood flow phenomenon—so called because of its unique railway boxcar aerial appearance—results ("BC" in Figures 1B and 2). Pioneering SCD investigators have used the conjunctival microcirculation in their research, speculating that the abnormalities were of significance not only because they could be detected but that they might reflect functionally and morphologically more deleterious or pathological microvascular events in the jeopardized tissues. These historical studies were based on slit-lamp biomicroscopy via 35-mm photography, and the results were mostly descriptive and rarely quantified.

It is normally not possible to directly study real-time microvascular abnormalities leading to soft tissue end organ damage in SCD patients. However, the readily accessible microvascular bed of the bulbar conjunctiva offers an excellent noninvasive site to extrapolate the in vivo microvascular condition at the soft tissue end organ level. We have used the conjunctival microcirculation in this study...
not only for ease of noninvasive access but also for clarity of image display and its uniqueness in being 1 of the 2 accessible microvascular beds in the human body with a complete, true capillary network. Furthermore, the same vessels can be easily identified and relocated for longitudinal investigations. Our laboratory has developed a novel computer-assisted intravital microscopy technology (intravital microscopy coupled with computer-assisted image analysis) to study diabetic microangiopathy and the changes (improvements) in the conjunctival microcirculation after successful simultaneous pancreas-kidney transplantations.20 We have adapted this noninvasive real-time technology to study and quantify the in vivo microvascular abnormalities in SCD.

Patients and methods

Patients and control subjects

SCD patients (ages 16-32 years) were recruited from the Sickle Cell Clinics at the University of California (UC) Davis Medical Center. Eighteen HbSS patients were studied, including 7 with severe and 11 with nonsevere SCD.
Table 1. Steady-state hematologic characteristics of the SCD patients

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Age, y</th>
<th>Hemoglobin, g/dL</th>
<th>Leukocytes, k/mm³</th>
<th>Platelets, k/mm³</th>
<th>Reticulocytes, %</th>
<th>F-hemoglobin, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients (from medical record)</td>
<td>35.80 ± 12.07*</td>
<td>8.22 ± 1.31†</td>
<td>12.72 ± 4.18*</td>
<td>323.64 ± 117.66*</td>
<td>10.68 ± 6.19†</td>
<td>31.6 ± 29.5‡</td>
</tr>
</tbody>
</table>

* n = 14.  † n = 12.  ‡ n = 7.
Eight successive video images were captured and used for each dynamic measurement of red cell velocity of a selected vessel in the field using VASVEL. Individual vessel diameter was also measured (using VASVEL) for velocity reference (eg, red cell velocity of 2.9 mm/s in a vessel with a diameter of 78.5 μm) and for single-vessel correlation with averaged values (using VASCAN).

Statistics

All objective measurements were averaged and reported as mean ± SD. Variables were compared using analysis of variance. A significance level of .05 was used. P values smaller than .01 (eg, .006 844 or 1.13 × 10⁻⁷) were presented as less than .01 for simplicity.

Results

Microvascular characteristics of non-SCD controls

Under intravital microscopy, the conjunctival vessels appeared as well-defined black lines and/or tubes on a white background (Figures 1-4). In the bulbar conjunctiva of the healthy non-SCD control subjects, we observed the orderly presence of an anastomosing network of capillaries, arterioles, and venules without the presence of any ischemic (avascular) zone (Figure 1A); these observations were in agreement with the classic definitive descriptions of Davis and Landau. The normal A/V ratio was about 1:2, and the arterioles and venules exhibited an even microvascular distribution without the presence of dilations, narrowing, microaneurysms, or sacculated vessels. Normal conjunctival blood flow, though variable in red cell velocity, was smooth and consistent (nonintermittent). The ischemic presentation of blood sludging, tortuous or arteriolesclerotic vessels, and the boxcar blood flow phenomenon, as previously identified by Davis and Landau for various vascular diseases, was rarely found in the healthy non-SCD (normal) controls. In this study, tortuous vessels were found in 2 non-SCD control subjects; we concluded that this occurrence was caused by contact lens usage and not vasculopathy (A.T.W.C., et al, unpublished data, 1996-1997).

Microvascular characteristics of SCD patients under steady-state conditions

Eighteen SCD patients (7 with severe and 11 with nonsevere SCD complications) were studied. Overall, the conjunctival microcirculation in steady-state SCD differed uniquely from the non-SCD conjunctival microcirculation. The orderly microvascular pattern of the normal bulbar conjunctiva was rarely seen, and abnormal microvascular features were easily recognizable in steady-state SCD patients (Figure 1B-D and Table 2). The A/V ratio was extremely variable and differed significantly (P < .01) from the normal non-SCD A/V ratio of about 1:2.

Under steady-state conditions, significant microvascular abnormalities (blood sludging, boxcar blood flow phenomenon, damaged vessels, distended vessels, tortuous vessels, and sacculated vessels) were identified in all 7 patients who had severe SCD complications (Table 2). The presence of comma signs and microaneurysms was identified in 5 of the 7 patients with severe SCD complications.

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Table 2. Microvascular abnormalities in steady-state SCD

<table>
<thead>
<tr>
<th>Steady-state microvascular characteristics</th>
<th>Patients with nonsevere SCD complications (n = 11)</th>
<th>Patients with severe SCD complications (n = 7)</th>
<th>Normal controls (n = 18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Comma signs</td>
<td>2</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Blood sludging</td>
<td>8</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Box car phenomenon</td>
<td>8</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>Damaged vessels</td>
<td>2</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>Distended vessels</td>
<td>2</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Tortuous vessels</td>
<td>7</td>
<td>7</td>
<td>2*</td>
</tr>
<tr>
<td>Sacculated vessels</td>
<td>6</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Microaneurysms</td>
<td>1</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

*These 2 controls were contact lens users. A study has shown that the large conjunctival vessels are tortuous around the edge of the contact lens (A.T.W.C., et al, unpublished data, 1996-1997).

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Table 3. Steady-state morphometric measurements of conjunctival vessels in SCD

<table>
<thead>
<tr>
<th>Experimental subjects</th>
<th>Total length of arterioles per area, cm⁻¹</th>
<th>Total length of venules per area, cm⁻¹</th>
<th>A/V ratio</th>
<th>Diameter of venules, μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCD (n = 18)</td>
<td>22.4 ± 8.7</td>
<td>24.6 ± 6.3</td>
<td>About 1:1</td>
<td>49 and 52</td>
</tr>
<tr>
<td>Non-SCD controls (n = 18)</td>
<td>26.8 ± 3.1</td>
<td>49.2 ± 6.7</td>
<td>About 1:2</td>
<td>55 ± 4</td>
</tr>
</tbody>
</table>

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Eight successive video images were captured and used for each dynamic measurement of red cell velocity of a selected vessel in the field using VASVEL. Individual vessel diameter was also measured (using VASVEL) for velocity reference (eg, red cell velocity of 2.9 mm/s in a vessel with a diameter of 78.5 μm) and for single-vessel correlation with averaged values (using VASCAN).
Eleven nonsevere SCD patients, who were relatively free of SCD complications, were found to have fewer manifestations of the above-listed microvascular abnormalities (Table 2). In 2 of the 11 patients with unremarkable SCD history (ie, with nonsevere SCD complications), the steady-state conjunctival microcirculation exhibited a minimal number of microvascular abnormalities, and the microcirculation in these 2 patients was comparable with non-SCD controls.

Morphometric measurements of SCD patients could be separated into 3 groups according to whether the averaged diameter of the conjunctival venules was the same as, significantly wider than, or significantly narrower than the non-SCD diameter (control) range (Table 3). In 2 patients with unremarkable histories of SCD, the averaged diameters of the large vessels (mostly venules) were comparable with non-SCD control values (49 µm and 52 µm, respectively; normal control range = 55 ± 4 µm). Twelve patients had venules with significantly wider diameter (79 ± 15 µm; P < .01) than the controls, with 6 of the 12 patients having severe SCD complications. Four of the remaining patients had venules with narrow diameters (40 ± 6 µm; P < .01) when compared with the controls; 1 of these 4 patients had severe SCD complications. In all 18 patients with SCD, a significant decrease in the presence of capillaries and small arterioles, an abnormal A/V ratio, and uneven vessel distribution were noted when compared with healthy non-SCD controls. In most SCD patients, the microcirculation of the bulbar conjunctiva diminished significantly, resulting in a blanched avascular appearance. At times, the bulbar conjunctival surface of some SCD patients showed an off-white grayish coloration, with multifocal dark lesions (damaged vessels and/or hemosiderin deposits).

**Intrapersonal variability of morphometric measurements in SCD patients under steady-state conditions**

Variability of steady-state microvascular features and measurements was minimal in the same patient(s) studied on different days. Intrapersonal variability in steady-state vessel diameter and total length(s) of vessels per area (objectively measured by VASCAN) was 4.1% ± 1.9% and 3.8% ± 2.8%, respectively, in 8 patients (selected at random from the 18 SCD patients) studied on separate steady-state visits scheduled 4 to 12 weeks apart.

**Red cell velocity under steady-state conditions**

In video sequences showing the conjunctival microcirculation, active blood flow was always visible in some, if not most, conjunctival vessels. However, red cell velocities in different conjunctival vessels varied considerably even in a short video sequence. To ensure this velocity variability was taken into consideration in data interpretation, red cell velocities in at least 5 vessels of each location (measured by VASVEL) were averaged. Then, the averaged red cell velocities of all patients were categorized into 3 red cell velocity groups (Table 4):

1. **Velocity 2.0 ± 0.4 mm/s in 10 patients with nonsevere SCD complications; differed but not significantly from non-SCD control values (2.5 ± 0.6 mm/s).**

2. **Velocity 1.4 ± 0.8 mm/s in 6 patients with severe SCD complications; differed significantly (P < .01) from non-SCD control values.**

3. **Intermittent trickle flow (≤ 0.2 mm/s) in 2 patients; 1 relatively free of SCD (nonsevere) complications and 1 with severe SCD complications. This trickled intermittent red cell movement during steady-state conditions differed significantly (P < .01) from steady-state SCD (both group 1 and group 2) and non-SCD control red cell velocities.**

**Acute and transient microvascular changes during painful crisis**

There was a significant decrease in conjunctival vascularity during painful crisis, with a close to complete absence of capillaries and a significantly reduced presence of arterioles and small venules from the steady-state conditions (Figures 3 and 4A). In most cases, the disappearance of capillaries and small arterioles arose as a result of the absence of blood flow into these microvessels. A few of the slugged large vessels did not show much change in diameter during crisis; however, most of the remaining large vessels showed an averaged decrease of 36.7% ± 5.2% in the diameter (compare Figures 4A and 4B).

Conjunctival red cell velocity, which was measurable in 16 of 18 SCD patients during steady-state conditions (velocity groups 1 and 2 in Table 4), either slowed significantly (46.6% ± 13.1% decrease in red cell velocity from steady-state values; P < .01) or was reduced to a trickle (≤ 0.2 mm/s or unmeasurable) when quantified within 5 hours after hospitalization for crisis treatment. These acute microvascular abnormalities were transient, and the microvasculature reverted to steady-state values after resolution of painful crisis (postcrisis). In reality, normal steady-state values are comparable to postcrisis values made 1 month after crisis resolution. During crisis resolution, reemergence of capillaries and arterioles (reperfusion) was observed in all patients (Figure 4B).

![Figure 3. A typical view of the conjunctival microcirculation in an SCD patient 1 hour after hospitalization for painful crisis.](Image)
Discussion

Using computer-assisted intravital microscopy technology, we have noninvasively identified significant in vivo microvascular (morphometric and dynamic) abnormalities in the conjunctival microcirculation of steady-state SCD patients. In this study, blood sludging, the boxcar blood flow phenomenon, damaged vessels, and abnormal vessel density and distribution were consistently present, albeit in varying degrees, in the conjunctival vessels in patients with severe SCD. These observations were significant because the presence of these microvascular abnormalities relates to blood flow impairment and the presence of ischemic zones in the bulbar conjunctiva.11-14 They also correlated with the general concept that vasculopathy normally underlies most of the complications and accounts for much of the morbidity and mortality in SCD.1,4,6 The arterioles and venules in some SCD patients were damaged, and hemosiderin deposits were present as multifocal lesions. In addition, vessel tortuosity and microaneurysms were found in some patients. Most of the microvascular abnormalities characterized in this study are in accordance with the classic definitive work of Davis and Landau23 who first described, via still photography studies, the presence of ischemic zones, blood sludging, boxcar blood flow phenomenon, microaneurysms, tortuous vessels, damaged vessels, and abnormal A/V ratio in vascular diseases. We have observed a unique decrease in overall vascularity, a change in A/V ratio, and abnormal distribution density of vessels in the conjunctival microvasculature under steady-state conditions. The presence of tortuous vessels, blood sludging, and abnormal A/V ratio is indicative of hypoperfusion and ischemia in the bulbar conjunctiva. These in vivo pathological conditions have been postulated to exist but have not been directly quantified previously.

The various microvascular abnormalities described above (with the exception of the comma signs and microaneurysms) were found in all 7 patients who were classified as having severe SCD complications and were sporadically found in some, but not all, of the 11 patients with nonsevere SCD complications (Table 2). We speculate that these microvascular abnormalities developed progressively due to tissue remodeling over the course of time and that these abnormal features collectively reflect the severity of the disease state. This study suggests that the sum total of the microvascular abnormalities found in the conjunctival microcirculation correlates with disease severity and clinical outcome of the SCD patients. To facilitate data correlation between disease severity, microvascular events (steady-state conditions and painful crisis), and hematologic findings, we are currently developing a quantitative Severity Index (computed as a summation of the collective presence of the 15 possible SCD microvascular abnormalities) that can be used by clinicians to assist in the objective evaluation of the clinical course of the disease or to follow disease progression and changes over time and during treatment.

Our work substantially extends earlier observations by other SCD investigators6-19,23 and offers several improvements. First, our technology represents the first utilization of a conjunctiva-dedicated intravital microscope design that is not based on the slit-lamp (biomicroscope) assembly previously used in other laboratories.6,9-19,23 The magnification and resolution of the optics are better than biomicroscopes, and the system is easy to operate. Coupling the intravital microscope system with videotaping capability offers additional cost, time, convenience, and reliability advantages. In addition, dynamic (red cell velocity) measurements can be conducted on videotapes, an opportunity that did not exist with still photography in historical studies. When combined, these improvements have enabled us to generate detailed and easily reproducible high-resolution images for morphometric and dynamic studies. The computer-assisted capability to frame-capture and objectively quantify (measure) the microvascular characteristics via image analysis further enhances the uniqueness of this technology.

Quantitative and noninvasive studies on blood flow in the in vivo human microcirculation have been limited. There are only 2 easily accessible sites for noninvasive in vivo microcirculation research in human subjects: the nailfold capillary bed and the conjunctival microcirculation. Lipowsky et al have utilized the nailfold capillaries in the fingers of SCD patients and have commented that the microvascular organization and vessel size in the nailfold microcirculation were not representative of the microcirculation at the organ/tissue level for relevant SCD interpretation.7 We used the conjunctival microcirculation because of its easy noninvasive accessibility, excellent quality of image display, and the reliability to relocate the same vessels for longitudinal assessment. In addition, the organizational and morphometric characteristics of the conjunctival microcirculation reflect more closely the characteristics of a true microvascular network (eg, normal presence and distribution of capillaries, arterioles, and venules) and are comparable with those of the microcirculation at the susceptible soft tissue end organ level in SCD.

Under steady-state conditions, 2 putatively healthy SCD patients were shown to have very slow or no blood movement.
(intermittent trickle flow) in their conjunctival vessels. This abnormal blood flow pattern differs significantly from normal non-SCD controls and other steady-state SCD patients with measurable blood flow (even in patients with severe SCD complications). One of the 2 patients was relatively free of SCD complications, and the remaining patient had severe SCD complications. Both patients were apparently healthy and not in acute painful crisis. This demonstration of steady-state conjunctival blood flow impairment was unexpected and difficult to explain. We believe that these 2 patients might have inherent cerebral vascular problems and that this conjunctival blood flow impairment might have been triggered by coexisting complications related to SCD.

We suspect that abnormalities in large intracranial vessels (eg, middle cerebral artery or internal carotid artery) coexist with abnormalities in small peripheral vessels (eg, conjunctival vessel) in SCD. We have also revealed a strong correlation (P ≤ .002, Fisher exact test) between severely compromised (intermittent trickle flow) conjunctival blood flow with high middle cerebral artery flow velocity (≥ 200 cm/s) and vulnerability to stroke in the same SCD patients in the reported study.

This study confirms that computer-assisted intravital microscopy represents the availability of a sensitive, repeatable, and quantitative real-time tool to study microvascular abnormalities in vascular diseases, including SCD. The utilization of the bulbar conjunctiva as a research site provides an ideal, readily accessible, noninvasive microvascular bed for in vivo research. Moreover, this site offers the additional advantage that the images of the conjunctival vessels are well resolved and easily identifiable for relocation in longitudinal studies. The fact that the experimental approach is objective and quantitative underscores the uniqueness of this technology and its potential as a clinical and/or research tool. Computer-assisted intravital microscopy (to study small vessel vasculopathy) can be used to correlate with transcranial Doppler ultrasonography or magnetic resonance angiography (to study large vessel vasculopathy) in the prediction of premature stroke in SCD. Intravital microscopy can be used to noninvasively study and identify key in vivo landmark microvascular events in different vascular diseases, including SCD, diabetes, and hypertension. The same objective and quantitative technology can also be used to monitor the efficacy of various therapeutic regimens in SCD, including chronic transfusion, hydroxyurea treatment, and allogeneic bone marrow transplantation. In each case, because the same vessels can be easily identified and relocated for repeated studies, the patient can reliably and appropriately serve as his or her own reference control for longitudinal data interpretation.

**References**


**Acknowledgment**

This manuscript is dedicated to the memory of the late Professor Benjamin Zweifach, a mentor and friend, who was instrumental in initiating 2 of us (A.T.W.C. and P.C.Y.C.) to develop the computer-assisted intravital microscopy technology and to study the conjunctival microcirculation.
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