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Vasculopathy, inflammation, and blood flow in leg ulcers of patients with sickle cell anemia

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Abstract

Chronic leg ulcers are frequent and debilitating complications of sickle cell anemia. Inadequate blood supply has been postulated to be an important factor in their occurrence and delayed healing. Little is known about their microcirculatory and histopathological changes. We evaluated the microcirculation of lower extremity ulcers with laser speckle contrast imaging and infrared thermography and obtained clinical and laboratory characteristics in 18 adults with sickle cell anemia and chronic leg ulcers. Skin biopsies were obtained in four subjects. Subjects had markers of severe disease, anemia, high degree of hemolysis, inflammation, and thrombophilia. The highest blood flow was present in the ulcer bed, progressively less in the immediate periwound area, and an unaffected control skin area in the same extremity. Microscopic examination showed evidence of venostasis, inflammation, and vasculopathy. Blood vessels were increased in number, had activated endothelium and evidence of thrombosis/recanalization. High blood flow may be due to chronic inflammation, cutaneous vasodilatation, venostasis, and in situ thrombosis. These changes in skin microcirculation are similar to chronic venous ulcers in the non-sickle cell disease (SCD) population, thus suggesting that leg ulcers may be another end-organ complication with endothelial dysfunction that appears in patients with SCD at a younger age and with higher frequency than in the general population.

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Author Contributions

CPM and KMD contributed equally to this work. CPM and GJK designed the research; acquired, analyzed, and interpreted the data; and wrote the paper. KMD and CRL contributed to the acquisition, interpretation, and analysis of data and writing of the manuscript. AMG contributed to research design, performed research, and interpreted some of the data. AK, NM, MA, and JM performed the imaging research and collected and analyzed data. DX analyzed data. SML contributed data and participated in editing the manuscript. EN contributed data and participated in editing the manuscript. MPL executed the research, and KCA performed essential part of the research.

Additional Supporting Information may be found in the online version of this article.

Introduction

Leg ulcerations are a serious and debilitating complication of sickle cell disease (SCD) and other hemolytic anemias associated with markers of severe disease and early mortality, both in the United States and other countries [1–6]. Their etiology and evolution is multifactorial and not completely characterized, but compromised blood supply secondary to vaso-occlusion has been considered a determinant factor in their occurrence and delayed healing [7–9]. Most recently endothelial dysfunction and thrombosis have been hypothesized to be contributing factors for this and other end-organ damage in SCD [10]. Current treatment options for leg ulcers are limited and outcome is unsatisfactory, with a high relapse rate and very slow rates of healing [1,12].

Patients with sickle cell anemia have vascular dysfunction and develop related complications, such as stroke, pulmonary hypertension, and renal dysfunction, at a higher rate than the nonsickle cell population. The potential role of vascular dysfunction in end-organ damage in sickle cell disease could be better investigated by physiological analysis of the regional microcirculation [12]. While this is virtually impossible to achieve in the intracranial or renal circulation, leg ulcers are more easily accessible and represent a unique opportunity to investigate blood flow and its alteration in patients with sickle cell disease.

Laser speckle contrast imaging (LSCI) and infrared (IR) thermography are new, noninvasive techniques useful in the in vivo study of skin and wound perfusion in animal models and humans [13,14]. The high resolution of LSCI offers an advantage over traditional laser Doppler imaging techniques and can image the entire physiologic range of blood flow velocities within a small diameter vasculature to a depth of approximately 300 μm [15]. These measurements reflect blood flow in capillaries, arterioles, venules, and dermal vascular plexuses and are sensitive to angiogenesis and vasodilation/constriction that occur during the different phases of wound healing [16]. IR measures tissue temperature up to 1 cm from the surface of the skin and its measurements correlate to forearm blood flow in patients with sickle cell disease [17].

We set out to study the regional blood flow of ulcer beds, and the immediate surrounding tissues compared to unaffected skin area in patients with SCD and chronic leg ulcers, in order to understand its role in ulcer pathogenesis and perpetuation. We also evaluated prospectively histopathology, markers of thrombophilia and inflammation, and detailed ulcer and patients' clinical characteristics.

Materials and Methods

Patients

All subjects were enrolled at the NIH Clinical Center on a clinical protocol approved by the institutional review board (ClinicalTrials.gov Identifier NCT01316796). Eligibility criteria required a subject to be at least 18 years old, have sickle cell disease, and have a leg ulcer of at least 4 weeks duration, between 2.5 and 100 cm^2 in size and not acutely infected. All evaluated patients were screened with a detailed medical history, physical examination, and clinical blood and urine tests. An echocardiogram was obtained in all subjects, as previously described [1]. The wound ostomy continence nurse (WOCN) service assessed the leg ulcers and obtained a wound culture. Care was taken to exclude an ulcer if it was acutely infected and or recently surgically manipulated. Laboratory evaluations were performed in the Clinical Center Department of Laboratory Medicine at the National Institutes of Health by standard clinical laboratory assays, including standard complete blood counts with hemoglobin F levels, thrombophilia markers, antinuclear antibody (ANA) and lupus anticoagulant, serum chemistry, and C-reactive protein (CRP). Patients were evaluated with

a detailed medical history, physical examination, echocardiogram, and routine laboratory studies obtained as needed to assess diagnosis, disease activity, and disease complications.

Imaging

One ulcer per patient was studied and is referred to as “study ulcer.” Images of the ulcer and surrounding region were obtained using a laser speckle contrast imager (LSCI, Full-Field Laser Perfusion Imager, Moor Instruments Ltd., USA) and infrared thermographic camera (IR, Santa Barbara Focal Plane, Lockheed Martin, USA). Prior to each imaging session, digital photographs were taken to be used for reference when drawing the ulcer border for selected LSCI regions of interest (ROIs), chosen to represent skin areas that appeared grossly distinct on clinical evaluation. For LSCI, these regions were located at the ulcer center (ROI 1), directly adjacent to the ulcer (ROI 2), and >5 cm away from the ulcer (ROI 3) as shown in Fig. 1. Measurements made by IR thermography encompassed the entire ulcer area (IR ROI 1), an annular region around the ulcer (IR ROI 2), and a distant region of the ipsilateral leg with intact skin (IR ROI 3). Optical images were collected for 30 minutes after 20 minutes of acclimatization at a room temperature of approximately 23°C. The cameras were directed toward the site of ulceration with the front lenses approximately 32 cm from the skin. LSCI blood flow measurements are reported in arbitrary units (AU) and represent the number of red cells detected in the image area per measured unit of time [18]. IR temperature recordings were in degrees Celsius and have a sensitivity of 0.015°C.

Pathology

Punch biopsies, 2–4 mm in diameter, of the skin were obtained from the edge of leg ulcers or symptomatic lesions in three patients. Tissue specimens were fixed in 10% neutral buffered formalin and routinely processed. Special stains including GMS, B&H, and AFB-FITE were performed on the tissue biopsies to exclude infection by fungal, bacterial, or acid-fast organisms.

Statistics

Summary statistics were used to describe the characteristics of the patients. For categorical variables, *P*-values were calculated using the Wilcoxon signed-rank test. Correlation analyses were conducted by Pearson’s method to detect linear correlation. Data analysis was performed with the use of R version 2.13.1 statistical software (2011-07-08) and GraphPad Prism version 5 (La Jolla, CA).

Results

Clinical and laboratory characteristics

Eighteen subjects with sickle cell anemia (homozygous SS), 8 men and 10 women, ages 20–59, with active chronic leg ulcers were enrolled in this study. Clinical and laboratory characteristics are available in the Supporting Information, as Table I.

Chronic opioid use was almost universal (16/18) and due mostly to localized pain at the ulcer site. Ulcers had been present for a median of 10 months (range 2–300 months) and treated with approximately eight therapies per patient. Half of the patients had one ulcer and half had more than one, up to 10 in one subject.

Eight subjects reported a prior diagnosis of pulmonary embolism (PE) or deep venous thrombosis (DVT) (two only PE, four only DVT, and two both) and five were on therapeutic anticoagulation at the time of the study. Thirteen of 18 subjects had an elevated factor VIII activity level (72%), one was heterozygous for prothrombin 20210 gene mutation (6%), and eight were positive for lupus anticoagulant (44%). Antinuclear antibody (ANA) was positive

in 8 of 18 subjects (44%). The results of the complete thrombophilia evaluation are shown in Table I. Of note, antithrombin III activity, which had been reported low in a series of SCD patients and leg ulcers, were low normal [19]. On average, mean arterial pressure (MAP) was low 62 ± 7.5 mmHg, anemia was severe (mean hemoglobin of 81 ± 1 g/L), LDH was elevated at 509.2 ± 247.3 U/L, and CRP and ferritin were very elevated at 87.6 ± 73.3 nmol/L and 2649 ± 4891 ng/mL, respectively. Zinc levels for this group were low normal, mean of 71.7 ± 12.1 mcg/dL (normal values: 66–110 mcg/dL), and four of them had abnormally low levels. Five of the eight males suffered from recurrent priapism (62%) and 11 of 18 had had previous episodes of acute chest syndrome (61%). Half of the patients were taking hydroxyurea and three were on chronic transfusions (two because of the leg ulcers and one for secondary stroke prevention). Renal dysfunction indicated by microalbuminuria was present in 9 of 18, or half of the subjects.

Echocardiogram revealed an elevated estimated pulmonary artery pressure indicated by tricuspid regurgitant velocity (TRV) >3.0 m/sec in four patients (22%) and >2.5 m/sec in 9 of 18 or 50% of the patients.

LSCI and IR thermography

Blood flow was greatest in the wound and periwound area compared to distant skin, as measured by LSCI red blood cell flux. A representative image of one patient's leg ulcer and the whole cohort's mean baseline blood flow for each ROI is shown in Fig. 1. The highest blood flow was measured in the ulcer center: $771.3 (\pm 479.9)$ AU, with progressively lower flow measurements moving away from the ulcer. LSCI was lower in the periwound: $455.6 (\pm 277.5)$ AU and in a distant area $136.9 (\pm 65.4)$ AU. These differences were statistically significant (overall $P < 0.01$, ulcer vs. peri-wound $P = 0.02$, periwound vs. unaffected area $P < 0.01$ and ulcer vs. unaffected area $P < 0.01$). Size and age of the ulcer did not correlate to blood flow, nor did the use of hydroxyurea, hemoglobin or percent sickle hemoglobin, CRP, or ferritin.

Further supporting the LSCI evidence of increased regional blood flow around the ulcer, periwound temperature was elevated compared to other regions, as measured by IR thermography. The mean measured temperatures in the ulcer bed, the immediate periwound area, and a distant region were $32 (\pm 1.6)$, $34.4 (\pm 1.1)$, and $33.9 (\pm 1.4)$ °C, respectively. The periwound area was significantly warmer than the distant skin region by a mean of 0.5°C ($P < 0.01$), suggesting increased blood flow in the periwound area. The periwound region was also significantly warmer than the ulcer bed ($P < 0.01$) as shown in Fig. 1, panel C. Ulcer beds have a moist surface that promotes evaporative cooling causing lower temperature than intact skin that is not indicative of blood flow. Periwound temperature was inversely correlated to ulcer size (Pearson correlation $r = -0.51$, $P < 0.03$).

No correlation was found between periwound IR temperature and hemoglobin, patient's age, use of hydroxyurea, or blood transfusions.

Histopathology characteristics

Skin biopsies were obtained in four subjects who consented to the procedure, three from the ulcer edge, and one from an area in the same extremity, but away from the actual ulceration. Histopathology showed a sharply demarcated ulcer edge (Fig. 2A). The base of ulcer was composed of granulation tissue with chronic inflammatory infiltrate and early scar formation. The epidermal changes adjacent to the ulcer were characterized by acanthosis, hyperkeratosis, and attenuated rete ridges (Fig. 2B). There were vasculopathic changes involving some of the small blood vessels subjacent to the base of ulcer, characterized by mural fibrin thrombi causing luminal narrowing and progressive vascular occlusion (Fig.

2D–F). The superficial dermal changes subjacent to intact skin were consistent with chronic stasis and vascular congestion, characterized by proliferation of small capillaries and venules with thickened vessel wall and patchy lymphoplasmacytic inflammatory infiltrate (Fig. 2C). There was an unusual proliferation of abnormal blood vessels with microangiopathic changes including fibrinoid necrosis in the vessel walls, and fragmented RBCs within vessel walls. One of the skin biopsies was obtained from a painful subcutaneous nodule located at the dorsum of right foot, at least 10 cm away from the ulcer at the ankle. Histologic examination revealed a subcutaneous hematoma. Further examination revealed vasculopathic changes involving a cluster of small blood vessels in the deep dermis (Fig. 2G). The lesional blood vessels showed eosinophilic fibrin deposits within the vessel wall associated with intimal hyperplasia and partial occlusion of the vascular lumen, findings indicative of vasculopathy (Fig. 2H).

Discussion

In this prospective study, we confirm and extend results from our group and others that patients with sickle cell anemia and leg ulceration have markers of particularly severe hemolytic anemia, including lower total hemoglobin and higher serum lactate dehydrogenase [1,20] than average sickle cell anemia patients. They also have severe disease, as demonstrated by a high incidence of complications, such as stroke, acute chest syndrome, thrombosis, renal disease, and elevated tricuspid jet regurgitation, when compared to data previously published obtained from patients with sickle cell disease without leg ulcers at our and other institutions [1,5,21,22].

Most patients reported daily opioids use, mostly for pain at the ulcer site rather than more typical vaso-occlusive pain, consistent with what has been previously reported [23]. This observation suggests that targeted analgesic interventions at the leg ulcer site might have a significant benefit in decreasing patient exposure to systemic opioids [24]. All patients' ulcers had been serially treated with numerous therapies, without sustained improvement, consistent with the lack of effective therapies and evidence-based guidelines on the best therapeutic options [11].

A prothrombotic predisposition is suggested by our data. Factor VIII levels were elevated in most patients; one patient was heterozygous for prothrombin gene mutation and most had borderline low levels of antithrombin III. Increased factor VIII levels have been linked with intravascular hemolysis and chronic inflammation and have been previously recognized to be elevated at steady state in many sickle cell patients [25]. The majority of patients reported a previous history of therapeutic anticoagulation for an earlier thrombotic event and over a third was positive for lupus anticoagulant, a potent risk factor for recurrent thrombosis in patients with a prior history of thrombosis. Few reports exist that focus on the microscopic morphology of skin biopsies in SCD patients with leg ulcers [26–28], and our study offers valuable new insights into their pathophysiology. We describe histopathological evidence of microthrombi and fibrinous deposition on luminal surface of the regional blood vessels in and around the ulcer, to further support the involvement of pathological hemostasis in the ulcerated tissue.

We found evidence of remarkably high blood flow in and around the leg ulcer bed, compared to a distant region of the same lower extremity, suggesting that slow healing is not merely due to absent/low skin perfusion. These data are in agreement with previous observations by Mohan et al. in Jamaica, comparing SCD patients with and without ulcers to normal controls [7]. High blood flow in the skin surrounding the ulcer is present in venous ulcers in the general population and is attributed to abnormal cutaneous flow caused by dilation, elongation, and tortuosity of capillaries of the skin [29,30]. The role of venous

incompetence in sickle cell leg ulcer formation has been controversial, suggested by some investigators [31,32] and dismissed by others [33,34]. Lower extremity swelling, induration, discoloration, and the clinical response to compression therapies frequently observed in leg ulcers in SCD patients, favor a role for venous hypertension in these lesions [2,11]. Our finding of increased number of blood vessels and fibrin thrombi with vascular occlusion, confirms the presence of venostasis in these patients. These changes are not intrinsic to the ulcer bed itself, as we observed in a region of the skin not directly part of the ulcer and may conceivably represent the vasculopathic changes that precede chronic ulcers in SCD.

Increased blood flow is part of the initial inflammatory and proliferative stages of normal wound healing in the general population. The increased flow promotes a vigorous immune response and neovascularization [35]. This phase typically occurs in the first 1–2 days after the wound has formed and is followed by apoptosis of newly formed blood vessels vascular endothelial cells and scar formation, with decrease in blood flow. Our results demonstrate that chronic, nonhealing leg ulcers in patients with SCD show signs of persistent inflammation and elevated blood flow, which suggest that healing is stalled in the inflammatory phase, much like chronic ulcer patients without sickle cell disease. It is conceivable that the high skin blood flow present in patients with SCD could constitute an etiologic step in ulcer formation, as increased blood flow associated with severe anemia and compensatory increased cardiac output in other vascular beds has been found to be associated with serious complications of SCD, preceding vasculopathy in stroke, and renal insufficiency [36,37]. Excessive blood flow causes pulmonary arteriopathy and pulmonary hypertension even without anemia or SCD [38]. Similarly, in our patients we observed evidence of severe vasculopathy, with blood vessel occlusion and partial recanalization, activated endothelium, thickened intima and distortion of the lumen, as well as increased number of blood vessels and evidence of venostasis.

Interpretation of these data is limited by the relatively small number of subjects examined, and by the fact that ulcers were analyzed months to years from their initial development. It would be ideal, but logistically difficult, to obtain microvascular blood flow data and histological material in the days or weeks immediately before ulcer formation. Therefore, our results focus on features that contribute to poor wound healing.

In summary, we report that in sickle cell patients, chronic leg ulcer beds and their immediate surroundings have high blood flow, underlying vasculopathy, venostasis, and thrombosis. Presumption of poor blood flow and consequent tissue ischemia has led to therapies aimed at increasing local perfusion and oxygen delivery using hyperbaric chambers, blood transfusion, and skin grafts, among the many interventions. Unfortunately, none of these therapies has been studied in a prospective randomized trial and anecdotal evidence for their benefit gives mixed results, with some reports of adverse events, such as hyperbaric chamber oxygen therapy apparently precipitating severe pain crisis [39] and chronic transfusion leading to iron overload. Interestingly, promising results were found with arginine butyrate [40] in sickle cell leg ulcers, a drug that could improve vasculopathy via either the arginine or the butyrate moiety, while hypercoagulability is a recognized risk factor for sickle cell [41] and nonsickle cell leg ulcers formation [42].

Interestingly, we find that ulcers in SCD patients bear strong similarities to chronic vascular ulcers in the general population, as far as the presence of high blood flow, abnormal blood vessels and chronic inflammation, at least during their active phase. This begs the question of whether there is such an entity as “sickle cell leg ulcer,” or there are ulcers that occur in patients who have sickle cell, the latter being a strong predisposing factor.

Our findings support involvement of vasculopathy in leg ulceration in SCD and may stimulate new conceptual models and investigation of therapeutic options.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Microvasculature analysis of ulcer area (ROI 1) and surrounding tissue (ROI 2) compared to a distant, nonaffected skin area (ROI 3). (A) Visible light photograph of the imaged ulcer area for a representative subject. Selected regions of interest are indicated with white squares. ROI 1 is the ulcer center, ROI 2 is the peri-wound area, directly adjacent to the ulcer, and ROI 3 is an area >5 cm from ulcer. (B) LSCI image of a representative patient and a graph that represents the average regional blood flows among 11 subjects. There is significantly different blood flow among the three regions of interest, with the highest mean blood flow in the ulcer center (ROI 1) and lowest distant from the ulcer (ROI 3) ($n=18$, $P<0.01$; paired Wilcoxon test; $P<0.01$). (C) Infrared image of the same representative patient. Temperatures as measured by infrared thermography in the ulcer bed (ROI 1), the peri-wound area (ROI 2) and a distant skin region (ROI 3). Temperature is highest in the peri-wound area compared to the ulcer bed due to evaporative cooling ($P<0.01$) and when compared to a distant area ($P=0.01$). Bar graph indicates mean values, and error bars indicate standard error of the mean, paired Wilcoxon tests.

Figure 2.

Microscopic analysis of skin biopsies. Evidence of increase in vascularity, chronic inflammation, vasculopathy with blood vessels occlusion, fibrin deposition in the intima, and microthrombi. Panel A. Scanning magnification view of the skin punch biopsy showing edge of an ulcer from the right ankle of patient MD. The epidermal changes adjacent to the ulcer are characterized by acanthosis, hyperkeratosis, and attenuated rete ridges. There is increased vascularity and inflammation in the dermis. (H&E, 100× original magnification). Panel B. The histologic changes subjacent to the ulcer bed are characterized by chronically inflamed granulation tissue with vasculopathic changes involving some of the small blood vessels. (H&E, 200× original magnification). Panel C. High magnification view of the superficial dermal vessels peripheral to the ulcer show proliferation of thick-walled capillaries and venules, consistent with chronic stasis. There is a lymphoplasmacytic inflammatory infiltrate in the dermis (H&E, 400× original magnification). Panels D, E, F. Very high magnification view of involved vessels subjacent to the ulcer bed reveals eosinophilic fibrin deposits within the vessel wall and partial occlusion of the vascular lumen (H&E, 600× original magnification). Panel G. Scanning magnification view of the skin punch biopsy obtained from the right dorsal foot of patient DD shows vasculopathic changes involving a cluster of small blood vessel in the deep dermis (H&E 40× original magnification). Panel H. High magnification view of the involved vessels reveals eosinophilic fibrin deposits within the vessel wall associated with intimal hyperplasia and narrowing of the vascular lumen (H&E, 400× original magnification).

TABLE I

Clinical and Laboratory Features of Participants ($n = 18$)

Variable	Value
Female sex (%)	56
Age (years)	39 $\pm$ 12
Ulcer size (cm ²)	4.1 $\pm$ 3.1
No. of ulcers (median, range)	1.5 (1–10)
Age of ulcers in months (median, range)	10 (2–300)
Hydroxyurea therapy (%)	50
Chronic transfusion (%)	17
Taking daily opioid (%)	89
Current anticoagulation therapy (%)	28
Hospitalization in past 12 months for vaso-occlusive crisis (%)	50
History of priapism (% of men)	63
History of pulmonary embolism or deep vein thrombosis (%)	44
Mean arterial pressure (mm Hg)	62 $\pm$ 7.5
White blood cell count ($\times 10^9/L$)	9.9 $\pm$ 4.5
Hemoglobin (g/dL)	8.1 $\pm$ 1.0
Platelets ($\times 10^9/L$)	332 $\pm$ 133
Absolute neutrophil count ($\times 10^9/L$)	5.1 $\pm$ 3.2
Absolute reticulocyte count ($\times 10^9/L$)	232 $\pm$ 181
Uric acid ($\mu\text{m/L}$)	7.9 $\pm$ 8.6
Aspartate aminotransferase (U/L)	46.6 $\pm$ 23.4
Alanine aminotransferase (U/L)	34.2 $\pm$ 18.2
Lactate dehydrogenase (U/L)	509.2 $\pm$ 247.3
Total bilirubin ($\mu\text{m/L}$)	42.8 $\pm$ 25.7
Creatinine ($\mu\text{m/L}$)	53.4 $\pm$ 15.3
Albumin, urine (mg/L)	133.1 $\pm$ 246.8
Ferritin (ng/mL)	2649 $\pm$ 4891
Pro brain natriuretic protein (ng/L)	134.4 $\pm$ 122.7
Zinc ($\mu\text{m/L}$)	10.97 $\pm$ 1.85
C-reactive protein (mg/L)	8.9 $\pm$ 6.8
Hemoglobin S (proportion of 1.0)	68.6 $\pm$ 26.6
Hemoglobin F (proportion of 1.0)	9.6 $\pm$ 8.9
Factor VIII activity (IU/dL)	250.2 $\pm$ 90.8
Protein C activity (% patients not on anticoagulants)	60.9 $\pm$ 17.3
Protein S activity (% patients not on anticoagulants)	61.5 $\pm$ 22.7
Factor V Leiden mutation analysis, positive (%)	0
Lupus anticoagulant, positive (%)	44
Antithrombin III activity (%)	80 $\pm$ 15
PT20210 mutation analysis, positive (n)	1
Antinuclear antibody, positive (%)	44

Clinical and laboratory results obtained when subjects were at steady state, without evidence of acute infection or pain crisis. A culture of the wound was obtained to demonstrate the absence of acute ulcer infection and showed normal skin flora.