Measurements of CD34+/CD45-dim stem cells predict healing of diabetic neuropathic wounds.

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Abstract:

Management of neuropathic foot ulcers in diabetic patients (DFUs) has changed little the past decade and there is currently no objective method to gauge probability of successful healing. We hypothesized that studies of stem/progenitor cells (SPCs) in the early weeks of standard wound management could predict who will heal within 16 weeks. Blood and debrided wound margins were collected for 8 weeks from 100 patients undergoing weekly evaluations and treatment. SPCs number and intracellular content of hypoxia inducible factors (HIFs) were evaluated by flow cytometry and immunohistochemistry. More SPCs entered the blood stream in the first two weeks of care in patients who healed (n=37) versus those who did not (n=63). Logistic regression demonstrated that blood-borne SPCs number and cellular content of HIFs at study entry and the first week follow-up visit predicted healing. Strong correlations were found among week-to-week assessments of blood-borne SPCs HIF factors. We conclude that assays of SPCs during the first weeks of care in patients with DFUs can provide insight into how well wounds will respond, and may aid with decisions on use of adjunctive measures.
**Introduction:**

The goal of this investigation was to determine whether circulating and wound margin stem/progenitor cells (SPCs) and intracellular contents of hypoxia inducible factors (HIFs) differed between diabetic patients with neuropathic foot ulcers (DFUs) that healed and those that failed to heal promptly with aggressive care. SPCs capable of multipotent differentiation can be mobilized from bone marrow and adipose tissue, enter the blood stream and migrate to peripheral sites where they may facilitate recovery from injuries (1). It has been estimated that SPCs contribute up to 25% of endothelial cells in newly formed vessels and by synthesizing growth factors they have a paracrine stimulatory impact on resident cell angiogenesis (2; 3).

DFU management is a major clinical problem and guidelines on the standard care of individuals with DFUs have changed little in the past decade (4; 5). There are no objective measures for prospectively evaluating the probability of success with standard treatment or for selecting adjuncts that may improve healing and lower the risk of amputations in those who fail to heal promptly. The success rate of standard therapy in randomized controlled trials involving subjects with adequate arterial flow in their lower extremities is only about 30% within 16 weeks of care (6).

SPCs mobilization to the blood stream occurs after wounding, physical exertion and in response to a variety of chemical agents (7-10). Clinical and animal studies provide evidence that SPCs are critical for neovascularization (3; 7; 11-15). Metabolic abnormalities associated with the diabetic state compromise SPCs characteristics and thus may contribute to healing impairment (7). A number of studies have demonstrated that wound healing can be improved by increasing
the number of circulating SPCs and/or enhancing wound site recruitment (2; 7; 16; 17). There also is evidence that some adjuncts to standard treatment such as negative pressure dressings and hyperbaric oxygen will mobilize SPCs to blood and may also modify regulatory protein content that improves vasculogenic function (11; 15; 18; 19). Pharmacological agents that might be used incidental to DFU treatment or as an adjunct may also influence SPCs (20-32).

Because many clinical variables may impact SPCs, attention to assessing their number or other characteristics may render many confounding variables manageable in evaluating healing potential. One recent report of 29 patients with DFUs found circulating SPCs with the surface marker CD34 and receptor for the vascular endothelial growth factor-2 decreased over a 12 week time span among those who healed (33). Animal studies have indicated that not only cell number but content of regulatory proteins such as HIFs influence vasculogenic potential (14; 34). A small clinical study suggested that insight into SPCs function may be gained by performing this analyses (15). However, there are technical challenges to measuring SPCs proteins, particularly when assays are performed at different points in time, because of heterogeneity that occurs as SPCs differentiate (35-38). Moreover, use of ‘housekeeping’ markers such as β-actin to normalize gene or protein content in these and some other cells has been called into question because measurements are so varied (35; 37; 39). HIF-1 and HIF-2 have ‘pro-neovascularization’ functions whereas HIF-3 negatively impacts SPCs vasculogenic function (34; 40; 41). Given these differences and because all three are regulated at the protein versus gene level, we investigated whether the HIF ratio (HIF-1 + HIF-2/HIF-3) measured in SPCs may predict wound healing.
The goal of this investigation was to determine whether patients who heal their DFUs within 16 weeks of care versus those who do not exhibit differences in circulating and DFU wound margin cells with surface markers that are currently considered consistent with SPCs, CD34-positive and CD45-dim (42).

**Research Design and Methods:**

*Study approval:* All subjects were patients presenting to the University of Pennsylvania Medical Center, Philadelphia, PA; or wound care clinics managed by Healogics, Inc. located at Roxborough Memorial Hospital, Philadelphia, PA or Greater Baltimore Medical Center, Baltimore, MD. The study was approved by Institutional Review Boards at each participating center and written informed consent was obtained from all subjects prior to inclusion in the study.

*Patient recruitment and management:* All patients had type 2 diabetes mellitus with a plantar surface foot wound due to neuropathy documented as loss of protective sensation by Semmes-Weinstein examinations and adequate arterial perfusion assessed as an ankle-brachial blood pressure ratio greater than 0.65 or skin perfusion pressure greater than 25 mmHg. In those patients with an ankle-brachial blood pressure ratio greater than 1.2 additional vascular evaluations were performed as outlined in (43). Exclusion criteria were: Inadequate arterial perfusion, institutionalization, previously received dialysis (peritoneal and/or hemodialysis) lasting more than one month, prior organ or bone marrow transplant; life expectancy less than three years as judged by the participant’s primary physician, immunosuppressive or other immunotherapy for renal disease within the past six months, self-report NYHA Class III or IV
heart failure at baseline, had received chemotherapy or alkylating agents for systemic cancer other than non-melanoma skin cancer within two years prior to enrollment, received treatment for systemic vasculitis that affects the kidneys, previous diagnosis of myeloma, known cirrhosis, HIV infection and/or AIDS based on participant self-report, currently participating in an interventional clinical trial, patient unable or not willing to comply with standard clinical practice guidelines or unable/ unwilling to provide informed consent.

Study subjects were approached when they first presented to wound clinics. The protocol spanned 8 weeks during which subjects returned to clinics for monitoring and wound care at approximately weekly intervals. At these visits blood was collected for SPCs analysis and when DFUs were sharply debrided, wound margins placed in 2% buffered formalin-filled vials for later analysis. All clinical management decisions were made by primary wound care providers; these decisions played no role in the study. Characteristics of the study population are shown in Table 1. Medications (listed in Supplementary Table 1) included: Insulin (n=85), statin drugs (55), one or more oral antibiotic (39), an ACE inhibitor (42), metformin (35), a beta-blocker (37), a sulfonylurea (24), a calcium channel blocker (19), an angiotensin converting enzyme inhibitor (42), clopidogrel (11), a steroid (3), a sympatholytic (2), a glucagon receptor blocker (4) and a PPAR gamma inhibitor (1). Specific wound care interventions are shown in Supplementary Table 2.

After the 8 week study protocol patients were monitored by maintaining contact with their wound care providers, as the primary outcome variable was whether a patient’s wound was healed by 16 weeks after study entry. Healing was defined as full epithelialization with the
absence of drainage and in those managed with split thickness grafting, as graft coverage/survival exceeding 95%. Two patients underwent a below the knee amputation and one a partial foot amputation prior to 16 weeks and were placed in the non-healed group.

Blood for SPCs analysis was collected in Cyto-Chex BCT test tubes (Streck, Inc., Omaha, NE) that contain a proprietary preservative. Samples were transported to the laboratory where analyses were performed within a time span of 1.5 weeks (preliminary work demonstrated that cell analysis is unchanged when studies are performed within 3 weeks on samples kept at room temperature or stored at 4°C). Formalin-fixed wound margins were typically shipped to the laboratory in formalin by overnight mailer, and always transferred to phosphate-buffered saline within 48 hours and stored at 4°C until immunohistochemistry analysis. As wounds improved, clinicians deferred debridements so fewer samples were analyzed in latter weeks. Similarly, when wounds healed within 8 weeks, on-going clinic care ceased and blood samples were no longer collected.

Materials: Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Antibodies for flow cytometry were purchased from the following sources: Brilliant Violet (BV)421-conjugated mouse anti-human CD34 and BV510-conjugated mouse anti-human CD45 from BD Pharmingen, San Jose, CA; R-phycoerythrin-(RPE) conjugated mouse anti-human HIF-1 from R & D Systems, Minneapolis, MN; fluorescein isothiocyanate (FITC)-conjugated mouse anti-human HIF-2 and Cy7- conjugated mouse anti-human HIF-3 from Bioss, Inc., Woburn, MA; allophycocyanin (APC)-conjugated mouse anti-human CD133 from Miltenyi Biotec, Auburn, CA. For immunohistochemistry the following antibodies were used: FITC-
conjugated mouse anti-human CD34 from Invitrogen, Grand Island, NY; RPE-conjugated mouse anti-human CD45 from Santa Cruz Biotechnology, CA; APC-conjugated mouse anti-human CD133 from Miltenyi Biotec Inc.; RPE-conjugated goat anti-human HIF-1 from R & D Systems, Inc.; A647-conjugated rabbit anti-human HIF-2 and RPE-conjugated rabbit anti-human HIF-3 from Bioss, Inc. Antibodies for Western blots were as follows: rabbit anti-human HIF-1, mouse anti-human HIF-2 and mouse anti-human HIF-3 from Abcam Inc., Cambridge, MA. Messenger RNA probes for VEGF type 1, Locus NM_003376 (VEGF) and SDF-1α, Locus NM_000609 (SDF-1) were purchased from Affymetrix eBioscience (Santa Clara, CA).

**Flow cytometry and cell analysis:** Most analyses were performed using an 8-color, triple laser flow cytometer MACSQuant (Miltenyi Biotec Corp., Auburn, CA) using the manufacturers’ acquisition software; early studies were done with a 10-color FACSCanto (Becton Dickinson, San Jose, CA). Flow cytometry was performed using 2 ml whole blood lysed with ammonium chloride, washed twice, permeabilized using saponin and incubated with antibodies following published techniques. Nucleated cells were segregated from debris using DRAQ5 DNA staining and gated based on CD34 and CD45 surface markers. The fraction of SPCs that expressed surface CD133 was also determined. All cell surface and intracellular marker analyses were performed utilizing the “Fluorescence Minus One (FMO) Control Test”. The merit of this approach has been well described by others and further discussion can be found in the On-line Supplement and in prior publications (14; 15; 34; 44). Total counted events/sample ranged from $2.2 \times 10^6$ to $3.2 \times 10^7$ so that from 252 to $2.8 \times 10^4$ SPCs/sample were counted. The HIF ratio was calculated as median fluorescence for HIF-1 plus HIF-2 divided by median fluorescence of HIF-3. On occasion, fluorescence for one intracellular protein was not detectable. When a
median fluorescence value for HIF-3 was 0, the HIF ratio was calculated by substituting the 0 with a value that was 10% below the lowest value recorded for the flow cytometer. Using a median fluorescence value of 0 for HIF 3, as reported by the cytometer, would make the HIF ratio infinite. Using a value that is 10% below the lowest value recorded for the flow cytometer reduces the calculated HIF ratio, but the value is a Real number. The modification also renders our analysis more conservative. As the same method was used for all data, our approach allows all values to be comparable. Expression of VEGF and SDF-1 mRNA were analyzed in CD34+/CD45-dim surface stained cells by flow cytometry using the FlowRNA II Assay kit (Affymetrix eBioscience, Santa Clara, CA) according to the manufacturer’s protocols (45). Representative histograms for flow cytometry analyses are shown in Results indicating overlaps between isotype controls and each marker of interest; thus components of some signals are not entirely specific.

**Western blot analysis:** CD34+/CD45-dim cells were isolated from blood using magnetic beads following published techniques (11). Isolated cells were suspended in PBS with 200 µM deferoxamine to impede HIF degradation, lysed and a total of 3 – 6 µg lysate protein loaded and separated on 4 – 12% polyacrylamide by electrophoresis followed by overnight transfer to a nitrocellulose membrane. The sheet was blocked with Odyssey blocking solution (Li-Cor Biosciences, Inc. Lincoln, NE, No. 927-40000) diluted 1:1 with PBS at 4°C overnight, and then incubated with primary anti-HIF (1:1000), HIF-2 (1:250) and HIF-3 (1:500) antibodies suspended in a 1:1 solution of blocking buffer plus 0.1 % Tween20 in PBS. Membranes were washed four times in 0.1 % Tween-PBS for 5 minutes under agitation and then incubated with appropriate secondary antibodies followed by treatment with enhanced chemiluminescence.
reagents (Amersham, Arlington Heights, IL, USA). Densitometric analysis (mean gray intensity) of gel films was performed with an Odyssey CLx Imager (Li-Cor Biosciences, Lincoln, NE). Positive identity of protein band locations was verified using cell lysates purchased from Abcam, Inc.

*Tissue staining:* Within 48 hours of formalin fixation, tissue was transferred to phosphate-buffered saline and rinsed for three 30 minute intervals. Samples were cryo-protected with three successive overnight incubations in 10%, 20% and then 30% sucrose before embedding in OCT cryo-medium, frozen at -20°C and cut into 20 µm thick sections. Images were analyzed on a Nikon inverted stage confocal microscope by first quantifying the background fluorescence. A minimum of three sections from each tissue sample were visualized with a total of 30-40 images taken per section. Each image reflected a wound tissue area of 225 µm². With each image, background fluorescence was first assessed so that positive staining could be defined as fluorescence of at least 150% over background. Then, using a standardized matrix for cell counting on each image, data were recorded as the mean number of CD34+ cells/image, the fraction of cells that also express CD45 (thus a calculation can be made for CD34+/CD45-dim cell recruitment), and fraction of CD34+ cells also expressing CD133. The fraction of CD34+ cells that contained HIF-1, HIF-2 and HIF-3 was also assessed.

*Statistics:* Patient age, gender, and hemoglobin A1C were summarized as mean ± SE; wound characteristics, SPCs enumeration and HIF ratio as median, 25th and 75 percentile. Correlations of measurements with healing by 16 weeks were evaluated by the Spearman rank order test. Log
transformations of SPCs, HIF ratio, wound size and patient age were used for logistic regression analyses. Sigmastat software (Systat, Point Richmond, CA) was used for data analysis.

Results:

Patient characteristics: Circulating and wound margin SPCs from 100 patients with neuropathic DFUs were studied. Table 1 shows general characteristics between patients who healed their wounds within 16 weeks from study entry (n=37) versus those who failed to heal (n=63). There were no statistically significant differences between groups for age, gender distribution, hemoglobin A1C or the duration of the wound prior to study entry. Wounds among those who did not heal had significantly larger volume at time of entry and after 8 weeks of wound care compared with those who healed. There were 8 patients in the non-healing group and 5 in the healed group (not significantly different, NS) with wounds that extended to tendon or bone at time of entry. No statistically significant differences between groups were present for medications or wound management interventions (Supplementary Tables 1 and 2).

Circulating SPCs analysis: SPCs values at time of study entry and the first week follow-up visit are shown in Table 1. Analyses were also performed adding the CD133 surface marker for early generation progenitor cells (33). Because this analysis followed the same pattern as SPCs and added little additional information, results are not shown. At study entry, the number of circulating SPCs was not statistically significantly different between those who healed and those who did not heal, but SPCs/µl blood increased significantly by the time of the first follow-up visit for patients who healed (Table 1). Thereafter, values at follow-up visits that occurred at approximately weekly intervals were not significantly different. Logistic regression analysis of
log transformed data found that the circulating SPCs number at the first follow-up clinic visit was associated with successful wound healing with an odds ratio (OR) of 3.9 (95% confidence interval [CI] 1.7, 8.9) (Table 2). No significant associations were found between use of medications (see Methods) and healing, nor was there an association between wound duration at study entry and healing. The wound size at study entry had a marginal predictive value with an OR 0.79 (CI 0.63, 0.98, p=0.03). When adjusted by including factors as described in Table 2 the values changed nominally: OR 0.73 (CI 0.57, 0.98, p=0.033). Including wound size in multiple logistic regression improved the predictive value for blood-borne SPCs number at study entry and the first follow-up visit (Table 2).

Because follow-up visits were not always performed at exactly 7 day increments, and at times patients missed appointments, we felt a more accurate way to evaluate SPCs changes over time was to compare follow-up values to those recorded at study entry and express results as the difference in number of SPCs/µl blood/day (Figure 1). Subjects who failed to heal within 16 weeks exhibited nominal change in circulating SPCs with early follow-up clinic visits (median difference at visit 1 was -0.0125 SPCs/µl blood/day), whereas numbers increased for those who healed (median difference at visit 1 was 0.0495 SPCs/µl blood/day). Values for those who healed were statistically significantly different from values for subjects who did not heal during the first two weeks of care. The correlation coefficient with healing for the difference in SPCs at the first follow-up visit was 0.58 (p<0.0001) and at the second visit, 0.29 (p=0.025).

The impact of medications on SPCs mobilization was performed by logistic regression using intake of a medication as the binary dependent variable. Metformin was the only medication
associated with a statistically significant impact on the SPCs value. At study entry the OR was 2.58 (CI 1.19, 5.59, p=0.017), at the first follow-up visit 2.75 (CI 1.19, 6.3, p=0.017) and at the second 2.56 (CI 1.19, 5.39, p=0.014). No significant association was noted for measurements from later visits. Analyses combining any other medication with metformin did not markedly change this result, nor were there significant findings with multiple medication analyses. Including metformin in the logistic regression model did not alter the effect estimate of SPCs on wound healing at study entry or the week 1 visit.

**HIF ratio:** As outlined in Introduction, we were interested in evaluating the cellular content of HIF proteins based on the ratio [(HIF-1 + HIF-2)/HIF-3]. There is little precedence for evaluating protein contents based on flow cytometry and whether findings with SPCs may be similar to more traditional Western blotting has not been investigated. Therefore, flow cytometry data were compared with results obtained using SPCs isolated using the magnetic bead technique from the blood of eleven patients that were lysed and proteins analyzed by Western blotting. Figure 2 shows three representative blots; there was a statistically significant association between HIF ratios quantified using flow cytometry median fluorescence values and Western blot band densities. In the Introduction problems with using ‘housekeeping’ proteins to normalize Western blots was discussed (35; 37; 39). We assessed β-actin variability within SPCs by placing lysates on the same blot to probe them in an identical fashion. The ratio of actin band density to number of cells used in each preparation was normalized to the densest actin band and values ranged from 8.7 to 100 %, with a median value of 52.4%. Thus, the use of a ‘housekeeping’ protein such as β-actin for standardization of flow cytometry data was abandoned in favor of using the HIF ratio. A representative flow cytometry analysis for SPCs HIF proteins is shown in Figure 3.
Median values for HIF-1, -2 and -3 as well as the HIF ratios for the 100 patient population are shown in Figure 4.

Logistic regression analysis of log transformed data found that the HIF ratios at study entry and the week 1 clinic visit were associated with successful wound healing (Table 2). The estimated odds ratio persisted in the fully adjusted analysis that included SPCs number, patient age and wound size at study entry. Interestingly, HIF ratios appeared to be relatively stable because values were highly correlated across the 8 weeks of study. Supplemental Table 3 shows correlation coefficients for the approximately weekly cell HIF ratios.

**Messenger RNA for VEGF and SDF-1:** VEGF and SDF-1 are among the HIF-dependent gene targets and the secreted proteins are critically involved with SPCs-dependent vasculogenesis. Therefore, we investigated the relationship between VEGF and SDF-1 mRNA species and HIF ratios in individual CD34+/CD45-dim cells from 9 subjects. Figure 5a shows a typical gating scenario and fluorescence signals for each mRNA species. Data (Figure 5b) were quantified as the fraction of cells showing positive staining, % VEGF and % SDF-1, based on FMO-analysis (see Methods) for each mRNA as well as median fluorescence. No significant associations between mRNA levels and HIF ratios were identified, but a weak association exists between % cells in the subjects’ blood exhibiting each of mRNA species (Figure 5c).

Stability of mRNA is obviously a concern with any fixed/stored samples sent from clinical sites. No relation was identified, however, between the duration of time from sample acquisition and mRNA levels. For example, a sample analyzed 1 hour after acquisition exhibited a median
fluorescence for VEGF of 7.6 and positive values found in just 2.8 % of CD34+/CD45-dim cells, whereas a sample analyzed more than seven months after acquisition exhibited a median fluorescence for VEGF of 6.9 and positive values found in 64.3 % of CD34+/CD45-dim cells.

**Wound margin immunohistochemical analysis:** The portions of wound margins obtained from sharp debridements containing full thickness epidermis were studied. Samples were analyzed for the number of CD34+ cells/image and the fractions of cells that also expressed CD45, CD133, HIF-1, HIF-2 and HIF-3. At study entry the median, 25th and 75th percentile number of CD34+/45-dim cells per image for patients who healed was 10.6 (2.4, 18.6) whereas for those who did not heal it was 6.1 (2.2, 16.2) (NS). Trends appeared for differences in fraction expressing specific HIF factors (e.g. median number of cells expressing HIF-1 at Week 1 among those who healed 5.8 (0.3, 18.5) versus 3.6 (0.8, 27.9) for the non-healing group) but variability was such that values were not statistically significant. The number of CD34+ cells/image and the fraction of cells expression HIF-1, -2, and -3 over the first 4 weeks of care are shown in Figure 6. Representative images from two subjects as well as the quantitative analysis of the images are shown in Figure 7. There were no statistically significant differences between groups across 8 weeks of sampling. However, within the collection of samples obtained from each patient the expression of HIF factors in wound margin cells were correlated. That is, when comparing the proportion of cells at a wound margin that expressed HIF-1, the correlation coefficients with fractions expressing HIF-2 and HIF-3 were typically between 0.5 and 0.7 (p<0.001, data not shown).

**Discussion:**
We believe this is the largest study to date examining associations between SPCs and clinical outcomes for DFUs healing. A significant increase in number of circulating SPCs occurs during the first weeks of wound care among patients who heal their wounds by 16 weeks. The circulating SPCs numbers in the first week of care can be used to predict wound healing. While some differences appear when SPCs are quantified using three surface markers (including CD133), trends pertinent to 16 week outcome are not impacted by this technical modification.

SPCs are mobilized in response to skin wounding and other forms of trauma (2; 8; 46). Sharp wound debridement was performed on all patients at their first clinic visit and we hypothesize this was the stimulus that triggered increases in circulating SPCs among healing patients. The number of circulating SPCs did not increase further after the first follow-up visit; possibly because fewer procedures or less aggressive debridements were performed as these wounds improved.

No increase in SPCs number occurred among patients who do not heal; in many cases a decrease was observed over weeks of care when changes were related to blood-borne SPCs numbers at time of study entry. The reasons why this occurred are not known. Glucose control was similar between the healing and non-healing groups and there were no notable differences in medications used. The proportions of subjects using metformin were not statistically significantly different between groups and its association with higher SPCs values is consistent with other reports (26). Wounds were larger in the non-healing group and size is a recognized risk factor for failure to heal (47). The predictive value of circulating SPCs improved when
adjusted for wound size. Whereas there is no precedence for thinking larger wounds would diminish SPCs mobilization, failure to mobilize SPCs clearly could contribute to larger wounds given the recognized role of SPCs with wound healing. SPCs from patients with diabetes exhibit impaired angiogenic function and patients have fewer circulating SPCs due to hyperglycemia, oxidative stress, inflammatory events and nitric oxide synthase phosphorylation in the bone marrow niche (13; 48; 49).

HIF proteins impact SPCs function and cell content can be quantified readily in animal studies (14; 34). We reasoned that a relationship among the HIF factors in SPCs may be linked with wound healing success. Because their activities are regulated mainly at the protein (versus gene) level due to hydroxylation/ubiquitination/proteolysis, we thought intracellular detection may prove meaningful. HIF-1 and HIF-2 are ‘pro-neovascularization’ factors (41). In contrast, based on protein depletion studies using small inhibitory RNA, HIF-3 adversely impacts vasculogenic SPCs function (34). Isoform 4 has a dominant-negative function of inactivating HIF1α-mediated transcription and one splice variant forms an abortive transcriptional complex with HIF-2α that prevents its gene interactions (40).

Our goal was to evaluate whether SPCs HIF ratios correlated with outcome. Ratios appear to have value for predicting healing within 16 weeks; but the functional relevance of the HIF ratio is unknown. Prior work has suggested that SPCs with higher content of HIF-1 and -2 are those primed for prompt mobilization from the bone marrow; these cells may be preferentially sequestered in the peripheral vasculature; and lower HIF levels in circulating SPCs might occur due to protein degradation (15; 19; 34). The similarities in HIF ratios across 8 weeks
(Supplemental Table 3) suggest that chronological age since mobilization and perhaps stresses sustained by the cells were similar. If true, then HIF ratio could reflect intrinsic differences in the SPCs populations between the healing and non-healing groups.

A weakness of this investigation is that functional testing of SPCs angiogenic potential was not performed. Hence, the results do not provide information on whether SPCs function varies among study participants. We have termed our CD34+/CD45-dim cells as SPCs, but we recognize that circulating endothelial cells are not excluded from the analysis. Measurements of intracellular HIF factors improves confidence that the cells of interest are indeed SPCs, however, because mature circulating endothelial cells would not be expected to exhibit measurable levels of HIF proteins (50; 51). There are many biochemical influences on HIF protein stability, HIF target gene transcription and stabilization of the mRNA that is produced. Any/all of these issues could explain why associations were not found between HIF ratios and mRNA levels for VEGF and SDF-1. Similarly, the duration of time that SPCs were in the circulation could also perturb a relationship. The half-lives for VEGF and SDF-1 mRNA are typically on the order of 1 hour and a variety of post-transcriptional controls have been identified (52; 53).

Surgical debridement of DFUs is intended to remove healing-impaired or senescent cells and thus render the wound more responsive to therapeutic interventions (54). As such, there are risks to using this tissue to glean insight into wound healing physiology. Our attempt was prompted by an earlier pilot study(15). Immunohistochemical analysis of wound margins can only provide semi-quantitative results and more detailed analyses with these clinically derived fixed tissue samples were not feasible. Our results did not demonstrate statistically significant differences
between those who healed and those who did not. Whereas some median values were markedly different, variability among the samples was high. We think it notable, however, that some sample-to-sample values, such as those for HIF expression, were highly consistent. Hence, further effort but using a larger sample size may provide information on SPCs at the wound site.

We conclude that assaying SPCs during the early weeks when patients are receiving wound care provides insight into how well wounds will respond. Whereas the absolute number of circulating SPCs in patients was small, the ~35% increase in cell count in the first week of care among those who healed (~0.05 SPCs/µl blood/day x 7 days, Figure 1) versus those who did not may provide useful clinical information that could influence wound care decisions. The relationships between SPCs number, as well as HIF ratio, and healing underscores the existence of quantifiable differences within the patient population. Further work is needed to determine whether analysis of SPCs number and/or HIF ratio can aid clinical decision-making. The annual incidence of foot ulcers among people with diabetes has been variously estimated at between 1% and 4.1%, and the annual incidence of amputation is 0.21–1.37% (5). Allocation of resources to achieve healing and when to incorporate more aggressive or adjunctive treatments early during the course of wound care requires objective measures. Blood-borne SPCs analysis may satisfy this need and also have utility in clinical efficacy trials where matching patients between various treatment arms obviously will impact results.

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Author contributions are as follows: SRT conceived ideas, oversaw the research program, performed laboratory studies, analyzed data and wrote the manuscript. MH aided with study subject recruitment, collected blood and tissue samples, collated data, reviewed the manuscript and provided advice. MAT aided with study subject recruitment, collected blood and tissue samples, reviewed the manuscript and provided advice. ZM aided with study subject recruitment, collected blood and tissue samples, reviewed the manuscript and provided advice. DSM aided with study subject recruitment, collected blood and tissue samples, reviewed the manuscript and provided advice. CD aided with study subject recruitment, collected blood and tissue samples, reviewed the manuscript and provided advice. OH analyzed data, reviewed the manuscript and provided advice. DW aided with study subject recruitment, collected blood and tissue samples, reviewed the manuscript and provided advice. MY performed laboratory studies, reviewed the manuscript and provided advice. KY performed laboratory studies, reviewed the manuscript and provided advice. VMB performed laboratory studies, reviewed the manuscript and provided advice. SK performed laboratory studies, reviewed the manuscript and provided advice. DJM conceived ideas, oversaw portions of the work, analyzed data, reviewed the manuscript and provided advice.
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Table 1. Characteristics of study population. Data show the number (N) of subjects in the group who healed and did not heal within 16 weeks and number who were female. Age (years), and hemoglobin A1c (%) are presented as mean ± SE. Wound (Wnd) duration (months), volume (cm$^3$) at study entry and after 8 weeks of monitoring, the number of circulating SPCs/µl blood in each group at time of study entry and at the first follow-up clinic visit are presented as median, 25$^{th}$ and 75$^{th}$ percentile; (*) denotes statistically significant difference between groups by Mann Whitney Rank Sum test.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th># Female</th>
<th>Age</th>
<th>HbA1c% (mM)</th>
<th>Wnd Duration</th>
<th>Wnd @ 8 wks</th>
<th>SPCs @ Entry</th>
<th>SPCs @ Wk 1</th>
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<tbody>
<tr>
<td>Heal</td>
<td>37</td>
<td>11 (29.7%)</td>
<td>58.4 ± 1.9</td>
<td>5.7 ± 0.3 (39.2 ± 3.3)</td>
<td>4.0 (1, 10.5)</td>
<td>0.36 (0.04, 0.87)</td>
<td>0.00 (0.0, 0.07)</td>
<td>0.98 (0.48, 1.54)</td>
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<tr>
<td>Not healed</td>
<td>63</td>
<td>16 (25.4%)</td>
<td>57.2 ± 1.5</td>
<td>6.5 ± 0.3 (47.8 ± 2.9)</td>
<td>4.0 (1, 10.5)</td>
<td>0.42 (0.18, 2.08)*</td>
<td>0.56 (0.09, 4.24)*</td>
<td>0.79 (0.62, 1.36)</td>
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</tbody>
</table>
Table 2. Association of SPCs characteristics with wound healing. Logistic regression was performed on log transformed data to assess the unadjusted odds ratio (OR) for healing using values of SPCs/µl blood and HIF ratio at study entry and the first week follow-up visit. Adjusted (Adj.) ORs were calculated by including values for SPCs number/µl blood, HIF ratio, patient age and wound size at study entry.

<table>
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<tr>
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<th>SPCs</th>
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<th>HIF Ratio</th>
<th></th>
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<td></td>
<td>Start</td>
<td>Week 1</td>
<td>Start</td>
<td>Week 1</td>
</tr>
<tr>
<td>OR</td>
<td>0.99</td>
<td>3.9</td>
<td>3.5</td>
<td>2.12</td>
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<tr>
<td>CI</td>
<td>0.54 – 1.82</td>
<td>1.7 – 8.9</td>
<td>1.5 – 8.2</td>
<td>1.2 – 4.3</td>
</tr>
<tr>
<td>P value</td>
<td>NS</td>
<td>0.001</td>
<td>0.003</td>
<td>0.021</td>
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<tr>
<td>Adj. OR</td>
<td>2.66</td>
<td>4.7</td>
<td>4.6</td>
<td>2.85</td>
</tr>
<tr>
<td>CI</td>
<td>1.11 – 6.34</td>
<td>1.8 – 12.0</td>
<td>1.8 – 12.1</td>
<td>1.3 – 6.3</td>
</tr>
<tr>
<td>P value</td>
<td>0.028</td>
<td>0.001</td>
<td>0.002</td>
<td>0.010</td>
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</table>
Figure 1. Change in circulating SPCs count relative to the count at study entry. Visits were scheduled weekly but subjects missed appointments on occasion or presented for care on alternative days, prompting expression of data as SPCs number/µl blood/day since entering study. Grey bars indicate data from patients who healed their wounds within 16 weeks versus those who did not (open bars). Data are median, boxes show 25th and 75th percentile, error bars show 10th and 90th percentile, with outliers shown as single dots. The (n) for each measurement is shown below the figure. Rank Sum p values between healed and not healed groups are shown for visits 1 and 2, values for visits 3 through 7 were not significantly different.
Figure 2. HIF ratio comparison between Western blotting and flow cytometry techniques. Representative SPCs Western blots (entire blots are shown) from three cell lysates (A, B and C). HIF-1 was monitored in the scanner 700 nm channel and HIF-2 and HIF-3 in the 800 nm channel. Anticipated molecular weight for HIF-1 was ~ 93 kDa, according to the antibody manufacturer HIF-2 typically exhibits two isoforms at 120 and 96 kDa, and HIF-3 molecular weight was 69 kDa). Figure at right shows linear regression comparison between HIF ratios calculated from flow cytometry versus Western blot data. These comparisons were made using cells from 11 study subject s.
Figure 3. Flow cytometry blood SPCs analysis for HIF proteins. After forward (FSC-H) and side scatter (SSC-H) interrogation, the P1 zone was selected and cells positive for CD34 and negative for CD45 defined based on the Fluorescence-Minus-One Control Test (box P3) were analyzed for content of HIF proteins. Histograms show fluorescence for cells incubated with antibodies to HIF-1, HIF-2 and HIF-3 as well as isotype control antibodies.
Figure 4. Fluorescence values for HIF proteins in blood SPCs. Data show median fluorescence, 25\textsuperscript{th} and 75\textsuperscript{th} percentile for CD34+/CD45-dim cell expression of HIF-1, HIF-2 and HIF-3; and the HIF ratio calculated as the median fluorescence of HIF 1 plus HIF-2 divided by HIF-3. The sample number for each value are as shown in Figure 1; there are no statistically significant differences between groups.
Figure 5. Measurements of VEGF and SDF-1 mRNA in circulating SPCs.

Figure 5a. Gating approach and typical quantification of mRNA. Top left figure shows laser light forward and side scatter with the lymphocyte population circled. Top right shows the lymphocyte population and the number of cells identified as CD34+/CD 45-dim based on FMO analysis (in the square 1.91 cells/µl were detected). Bottom two histograms show fluorescence values (abscissa) and number of events (ordinate) for SDF (left) and VEGF (right) mRNA along with the FMO control.
Figure 5b. Relationships between mRNA for SDF-1 and VEGF with HIF ratio. Data show results for 9 cell samples with HIF ratio on the abscissa, % CD34+/CD45-dim cells exhibiting mRNA-specific fluorescence above the FMO baseline as closed circles on the left ordinate axis, and median mRNA fluorescence as open circles on the right ordinate axis. There are no statistically significant associations between mRNA types and HIF ratio.
Figure 5c. Relationship between % of cells positive (above the FMO baseline) for SDF-1 mRNA and VEGF mRNA.

\[ R^2 = 0.46 \]
\[ p = 0.045 \]
Figure 6. Immunohistochemistry results. Data from weekly analyses are shown for number of CD34 cells/image (left panel) and the fraction of cells expressing HIF-1, -2 and -3 (right three panels) defined as those cells exhibiting a fluorescence value of at least 150% over background. Data are displayed as described in Figure 1 and the (n) for each sampling is the same as in Figure 1. Analyses involved imaging a minimum of three 225 µm² tissue sections from each patient, with a total of 30-40 images taken per section. There were no statistically significant differences between patients who healed within 16 weeks (noted as H at the bottom) and those who did not heal (NH).
Figure 7. Representative confocal microscope images of wound margin tissue from two patients at week 1 follow-up visit; (A) is from a patient who healed, (B) a patient who did not heal within 16 weeks. One set of tissue sections was stained with antibodies to CD34 as well as HIF-1 and HIF-2, another with CD34 and HIF-3; merge images depict overlap between respective protein staining. The table beneath the images show the quantitative analysis for the sections as median CD34+cells/image and % of cells exhibiting fluorescence for HIF-1, HIF-2

<table>
<thead>
<tr>
<th>CD34/image</th>
<th>%HIF1</th>
<th>%HIF2</th>
<th>%HIF3</th>
</tr>
</thead>
<tbody>
<tr>
<td>40.47</td>
<td>46</td>
<td>67</td>
<td>61</td>
</tr>
</tbody>
</table>
and HIF-3. The orientation for all samples was epidermis toward the top of the image.
<table>
<thead>
<tr>
<th>CD34/image</th>
<th>%HIF1</th>
<th>%HIF2</th>
<th>%HIF3</th>
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<tbody>
<tr>
<td>10.73</td>
<td>91</td>
<td>82</td>
<td>92</td>
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Supplementary Information on Flow Cytometry: Blood cells were gated based on forward and side laser light scattering, and nucleated cells were segregated from debris by DRAQ5 DNA staining. As discussed in the Methods section of the paper, the majority of studies were performed using a triple laser flow cytometer MACSQuant (Miltenyi Biotec Corp., Auburn, CA) that can simultaneously detect up to 8 colors using the manufacturers’ acquisition software. Fluorescence signals from the three (404, 488 and 635 nm) excitation lasers using five band-pass filters (450/50-nm, 525/50-nm, 585/40-nm, 655-730-nm and 750 nm LP) were quantified based on the Fluorescence-Minus-One (FMO) method to determine cytometer boundaries for acceptance of an event as a SPCs and potential marker interactions. The approach removes operator-dependent judgements for selecting gating parameters and provides an objective way to place the boundary for non-staining cells in a channel versus an arbitrary pre-set decision of acceptance based on a signal-to-noise ratio (44). Because the compensation corrections differ in part based on amounts of the various reagents within cells in different subsets (e.g. those used for the various HIF measurements) use of FMO determines the boundary between positive and negative cells for each subset. Representative flow cytometry analyses are shown in the text and are analogous to those described and illustrated in prior publications (14, 15, 34).

Supplementary Table 1. Medications patients taking during the study. Subjects had been taking their medications prior to study entry and there were no deletions or additions during the study interval. Medications listed include insulin, metformin (Met.), sulfonylureas (Sulf), angiotensin converting enzyme inhibitors ACE), angiotensin receptor blockers (ARB), calcium channel blockers (C++B), β-blockers (β-b), statin agents, clopidogrel (Clopid), a steroid and various ingested antibiotics (Ab).

<table>
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<tr>
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<th>Insulin</th>
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<th>Sulf</th>
<th>ACE</th>
<th>ARB</th>
<th>Ca++B</th>
<th>β-b</th>
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<tr>
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<td>10</td>
<td>6</td>
<td>13</td>
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<td>6</td>
<td>11</td>
<td>18</td>
<td>4</td>
<td>0</td>
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<td>(37)</td>
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</table>
Supplementary Table 2. Wound management. In addition to materials shown in the table, during the 8 week interval of the study wounds for one person in each group (healed and not healed) were treated with a compression dressing, negative pressure wound therapy, hyperbaric oxygen therapy and a cell-based skin substitute. Two in the healed group received a honey-based dressing and two in the non-healed group had becaplermin applied.

<table>
<thead>
<tr>
<th></th>
<th>Iodo- or Silver-ointment</th>
<th>Carboxymethylcellulose (CMC)</th>
<th>CMC +collagen + silver</th>
<th>Collagenase ointment</th>
<th>TCC</th>
<th>Antibiotic Ointment</th>
<th>xenograft</th>
<th>Extracell. matrix</th>
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<td>4</td>
<td>8</td>
<td>4</td>
<td>6</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>2</td>
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<tr>
<td>Not heal (63)</td>
<td>27</td>
<td>7</td>
<td>6</td>
<td>9</td>
<td>7</td>
<td>9</td>
<td>10</td>
<td>5</td>
<td>4</td>
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**Supplementary Table 3. Correlation coefficients for intracellular HIF ratios.** The ratio, (HIF-1 + HIF-2)/HIF-3 was evaluated for circulating SPCs defined as CD34+ and CD45-dim. The (n) for each measurement is the same as shown in Figure 1 caption. Where p values are shown, correlations are statistically significant when corrected for multiple comparisons and these values are in bold.

<table>
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<th>Day 1</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
<th>Week 5</th>
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<th>Week 7</th>
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<td>-0.440</td>
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<td></td>
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<td>0.597</td>
<td>0.560</td>
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