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Circulating endothelial precursor cells are associated with a healed diabetic foot ulcer evaluated in a prospective cohort study

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Abstract

Objective: The goal of this study was to evaluate whether circulating endothelial precursor cells (CEPCs) and neutrophil microparticles (MP) can predict diabetic foot ulcer healing.

Research Design and Methods: A multicenter study was designed to evaluate circulating cellular markers, CEPCs and MPs, as prognostic factors associated with the healing of DFU by the 16^{th} week of care. Flow cytometry analysis of CEPCS and MPs were obtained at the first visit and compared to wound healed status.

Results: 207 subjects were enrolled at four sites. 40.0% (28.4,41.5) of the subjects healed by the 16^{th} week of care. Several CEPCs measured were associated with healing after adjustment for wound area and wound duration. Typical of this analysis was $CD34^{+}CD45^{dim}$, the univariate OR was 1.19(0.88,1.61) and after adjustment for wound area and wound duration the OR was 1.67(1.16,2.42) p=0.006). A prognostic model with CEPCs $CD34^{+}$ $CD45^{dim}$, wound area, and wound duration had an area under the curve (AUC) of 0.75(0.67, 0.82) and, simpler, $CD34^{+}$

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Author contributions

DM, DSM, ZM, JL, HLT, RK, DR, VB, ST obtained data from subjects or their specimens. DJM, OH, and NM analyzed the dataset. DJM wrote the first draft of the manuscript. DM, NM, OH, DSM, ZM, JL, HLT, RK, DR, VB, ST revised/edited the manuscript and accepted the final draft of the manuscript.

Conflict of interest and Duality of Interest:

The authors report no conflicts of interest with respect to the topic of this study.

$CD45^{dim}$ per initial wound area, as a solitary predictor, has an AUC of 0.72 (0.64, 0.79). MPs were not associated with a healed wound.

Conclusions: Previous studies have indicated that CEPCs measured at the first office visit are associated with a healed DFU. In this multi-centered prospective study, we confirm this finding, show the importance of adjusting CEPCs measurements by wound are, and show that a single number based on CEPCs per wound area is highly predictive of a healed DFU by 16^{th} week of care.

Introduction

We carried out a multicenter clinical investigation to evaluate the relationship between resolution of patient's diabetic foot ulcer (DFU) and the number of circulating endothelial precursor cells (CEPCs), microparticles (MPs) and leukocyte content of Nitric Oxide Synthase (NOS)-one accessory protein (NOS1AP). The goal was to assess whether one or more laboratory markers might be associated with a DFU healing by the 16^{th} week of care that could ultimately be used to improve our ability to predict this clinical outcome. DFU and lower extremity amputation (LEA) are devastating complications of diabetes mellitus (DM) that carry an annual risk of mortality of about 10–20% with per person cost of $10,000 to $60,000 per annum.(1–4) Individuals with DM develop LEA for many reasons. About 90% of individuals with LEA have histories of foot ulcers and have lower extremity findings consistent with peripheral arterial disease (PAD) and/or diabetic peripheral neuropathy (DPN).(1; 2) Of those who have a LEA, about 34% will have a second more extensive LEA within 16 weeks of their initial amputation.(5) Persistent DFU are by defined as wounds that have failed to heal in a timely manner.(6) Those who develop chronic wounds that result in LEA have wounds that heal slowly or not at all. Why a wound fails to heal is not well understood. Many hypotheses have been formulated including ones that describe an essential role for neovascularization.

CEPCs constitute about 25% of endothelial cells that are part of newly formed vessels and serve a paracrine role in tissue regeneration by liberating an array of proteins and nucleic acids that are often contained within secreted extracellular vesicles. (7–9) We previously evaluated natural variation in the *NOS1AP gene* and variation in CEPCs numbers with respect to the healing of DFU as well as the onset of LEA.(10–16) Others have shown that CEPCs are reduced in individuals with DM.(17) We previously demonstrated that individuals with DFU are more likely to heal if they have an increased number of CEPCSs in the first weeks of wound care.(15) The adjusted odds ratios (OR) of association were 2.7 (1.1, 6.3), p=0.028 and 4.7 (1.8, 12.0) p=0.001, at the start of and at the first week of treatment, respectively.(15) We sought to confirm these findings prospectively in a larger cohort.

The NOS classes of proteins synthesize NO that has been shown to be an important cell signaling molecule with respect to angiogenesis and wound repair.(18; 19) In humans, single nucleotide polymorphisms (SNPs) in *NOS1AP* have been associated with DM (although the association was not confirmed in validation studies), cardiac arrhythmia, schizophrenia, and LEA.(20–22) The mechanism of action of NOS1AP on wound repair is not understood.

We have identified a role for the NOS1AP protein, which is also called Capon, with generation of inflammatory MPs in response to hyperglycemia(23). MPs are 0.1 to 1 μm diameter extracellular vesicles formed by cell membrane evagination.(7) These particles can be generated by CEPCSs and myriad other cell types, they are 2–10 fold elevated in those with DM and may serve pro- or anti-inflammatory roles. MPs from patients with metabolic syndrome, but not healthy subjects, induce vascular dysfunction in *ex vivo* studies, as well as when injected into mice (24; 25).

Understanding the likelihood that a DFU might heal is critical in the clinical care of patients. Models developed from large administrative databases, cohort studies, and randomized clinical trials have been used for risk stratification and clinical prediction based on first visit assessment of the size and the duration of the wound.(26–28) The goal of this study was to evaluate whether CEPCs, MPs, and leukocyte NOS1AP levels can improve the prediction of DFU healing.

Methods

Cohort

A multicenter study, called the Diabetic Foot Ulcer Consortium (DFUC), was designed to evaluate circulating cellular markers, CEPCs and MPs, as prognostic factors associated with the healing of DFU.(29) The DFUC also collected routine clinical data.(29) The DFUC is composed of wound care centers at academic institutions, University of Miami, Icahn School of Medicine, and University of Pennsylvania, as well as the community based-MVS Wound Care in Maryland. The goal was to enroll 200 subjects to achieve 80% power to be able to detect an odds ratio of 1.4 (expected OR of association for $CD34^{+}$ $CD45^{dim}$ cells from previous study)(15) assuming that the probability of healing was 0.40. All subjects were examined by a collaborator/local investigator from the wound care centers, had history of adult-onset DM, were at least 40 years of age at the time of original DFU diagnosis, and, per the local investigator, had a physical examination consistent with DFU, had adequate arterial flow for healing, and had a DFU on the plantar aspect of the foot that was eligible for standard care. Standard care routinely included a history and physical examination including evaluation of lower extremity arterial flow (e.g., palpation of foot pulses, arterial brachial index, etc.), assessment of sensory neuropathy (e.g., Semmes Weinstein monofilaments), sharp debridement, off-loading (e.g., total contact cast, removable walker, etc.), treatment of infection (if present), a primary bandage, and recurring periodic evaluation. As part of standard care, based on progress over the first few weeks, change in the treatment plan including surgery or other therapies could be considered. The study outcome was a healed wound by the 16^{th} week of care. All subjects signed a consent form approved by the appropriate Institutional Review Board.

Lab methods

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Antibodies were purchased from the following sources: Brilliant Violet 421–conjugated mouse anti-human CD34 (cat # 562577), Brilliant Violet 510–conjugated mouse anti-human CD45 (cat # 563204), PerCP-Cy5.5 mouse anti-human CD90 (Cat # 561557), APC mouse

anti-human CD117 (Cat# 313206), and PE/Cyanine7 mouse anti-human CD146 (Cat #342010) from BD Pharmingen, San Jose, CA; Alexa 488 phalloidin (Cat# A12379) from InVitrogen (Waltham, MA); rabbit anti-NOS1AP (Cat# 190686) from Abcam (Waltham, MA).

Flow cytometry analysis of CEPCs and MPs followed methods as described in our previous publications (15; 23; 30). In brief, total MPs and the sub-types were assayed using standard flow cytometry methods including a fluorescence minus one control step to enumerate 0.3–1.0 μm diameter particles that bind annexin V (indicative of exterior membrane phosphatidylserine). Surface markers were assayed that are linked to bone marrow-derived stem-progenitor cells (CD34+/CD45-dim), CD117 (stem cell factor receptor) and CD90 (marker of many stem cell populations and less prominently on endothelial cells) while selecting against the endothelial cell marker (CD146). An additional component of the study was to examine phalloidin binding to MPs. This was done because of recent study suggesting that some inflammatory MPs expressed filamentous actin on the membrane surface.(30) Western blot analysis for NOS1AP was evaluated in leukocytes isolated from blood and normalized to cell content of β-actin following previously published methods (23).

Analysis

We previously described the analysis of clinical wound factors in DFUC.(29) For this analysis, we focused on CEPCs, MPs, and serum capon levels. Many of these factors were not normally distributed so they were natural log transformed. Linear or logistic regression was used to assess the strength of association between each potential prognostic factor and the 16-week healing outcome. We then developed a prognostic model that we evaluated for discrimination using the area under the receiver operating curve (AUC). The calibration of the model was evaluated using Hosmer-Lemeshow goodness-of-fit statistics. We compared our model to those from previous studies that focused on wound area and wound duration. Additionally, we used LASSO (**L**east **a**bsolute **s**hrinkage and **s**election **o**perator), a machine learning algorithm, to build a prediction model. LASSO is an adaptive regression-based approach for high-dimensional data that uses 10-fold cross validation.(31)

Results

The DFUC enrolled 207 subjects. Basic information about his cohort has been previously published and presented in Table 1.(29) Subjects were enrolled at four sites (percent of total enrollment): University of Miami (7.8%), Icahn School of Medicine (16.2%), University of Pennsylvania (47.6%) and MVS Wound Care in Maryland (28.4%). The average age at enrollment was 57.8 (56.4, 59.1) years, 94.2% had adult-onset DM with an average age of onset of 39.4 (37.5,41.4) years, 73.0% were men, 58.3% were Black and 36.8% were White. While 40.0% (28.4,41.5) of the subjects healed by the 16th week of care (the primary outcome), healing rates varied significantly by site (28.9%, 51.7%, 25.0%, 30.3%, respectively; p=0.02). The variation in wound healing rates by center is most explained by variations of the wound size and wound duration at the time of enrollment.(29)

Three blood-based factors were measured: CEPC (as identified by a variety of cell surface markers with our primary measure being $CD34^{+}$ $CD45^{dim}$), circulating MPs (as measured by a variety of cell surface markers), and the NOS1AP protein (which was measure per unit of actin protein in leukocytes) were evaluated with respect to a healed wound by the 16^{th} week of care (Tables 2 and 3). Several of the measures of CEPCs were statistically significant after adjusting for wound area and wound duration (Table 3). For example, for our primary CEPCS measure, $CD34^{+}CD45^{dim}$, the univariate OR was 1.19(0.88,1.61) and after adjustment for wound area and wound duration the OR was 1.67(1.16,2.42) p=0.006) (Table 3). Wound area is likely the primary confounder (1.45(1.03,2.03) p=0.032) (Tables 1 and 2). This association is not confounded by duration or age of onset of DM, anatomic depth of the wound, sex, age, eGFR, neuropathy, race, BMI, and ABI.

To better understand the effect of wound size and wound duration on CEPC number, associations were investigated using linear regression. The overall number of CEPCs increases (0.071(−0.004,0.145) p=0.063) and the number of cells appears to decrease as the wound duration increases (−0.013 (−0.142,0.115). Finally, as a dichotomous predictor, $CD34^{+}$ $CD45^{dim}$ > 20 cells per 100,000 WBC per μL of blood is associated with increased likelihood of healing (2.16(1.09, 4.29) p=0.028). Less mature CEPCSs (CD34+/$CD45^{dim}$/$CD146^{dim}$, CD34+/CD117+/$CD45^{dim}$/146^{dim}) were also associated with an increased odds of healing after adjustment for wound size (Table 3). Associations were also found for cells expressing proteins associated with mesenchymal stem cells ($CD34^{dim}$/CD117+CD90+ and $CD34^{dim}$/CD117+/CD146+).

As a predictor of wound healing, CEPC measurements are not significantly associated with the commonly used surrogate markers for a healed wound by the 16^{th} week of care; such as percentage change in wound area in the first four weeks or the achievement of a 50% reduction in wound area by the 4^{th} week of care.(26; 27; 32) For example, the association of $CD34^{+}$ $CD45^{dim}$ to percent change in wound area in the first four weeks is not statistically significant (linear regression coefficient 0.51 (−0.24,1.26) p=0.181). The association is not influenced by initial wound area (0.26(−0.48,1.00) p=0.488.) In addition, using the dichotomous outcome of a 50% reduction in wound area by the 4^{th} week of care also showed no significant association ((1.11(0.81,1.54) p=0.513 or 1.25(0.88,1.75) p=0.212) after adjustment for initial wound area). However, many of the variables listed on Table 2 are excellent predictors of whether a wound heals by the 16^{th} week of care. As a base line, a frequently used prognostic model includes the natural log of wound area and wound duration. In these predictors have an AUC of 0.72 (0.64,0.80) with respect to a healed wound by the 16^{th} week of care. By itself log transformed $CD34^{+}$ $CD45^{dim}$ is not a great predictor with an AUC of 0.55 (0.46,0.63). However, the addition of log transformed $CD34^{+}$ $CD45^{dim}$ to log wound area and log wound duration improves the AUC to 0.75(0.67, 0.82) and log transformed $CD34^{+}$ $CD45^{dim}$ per log transformed initial wound area, as a solitary predictor, has an AUC of 0.72 (0.64, 0.79). Furthermore, using LASSO regression with the factors on Tables 2 and 3 as well as factors such as wound area, wound duration, and wound depth, resulted in a final LASSO model with a single predictor log transformed $CD34^{+}$ $CD45^{dim}$ per log transformed initial wound area.

Due to technical issues only 131 Capon/actin measures were available for analysis. A significant association was not noted for leukocyte capon content (univariate OR 0.97 (0.78, 1.20), p=0.78; or adjusted area ln duration ln OR 0.9 (0.71, 1.14), p=0.37 with respect to a healed wound by 16th week of care. This may be due to the small sample size. However, as might be expected given that capon is associated with an inflammatory response(33), capon/actin using linear regression is inversely associated with CD34^{+} CD45^{dim} (−0.38 (−0.74, −0.02) p= 0.034). Additionally, no associations were noted for circulating microparticles expressing the various combinations of CEPCSs surface proteins (Tables 2 and 4).

Discussion:

CEPCs, as defined by CD34^{+} CD45^{dim} cells, measured at the commencement of care are associated with a DFU healing by the 16th week of care. Individuals with DFU who have larger number of CEPCSs are the most likely to heal. The number of CEPCSs is associated with the size of the wound. Most DFUs are less than 5 cm^2 and for these individuals the presence of more than 20 CEPCSs (CD34^{+} CD45^{dim}) per 100,000 WBC per μL of blood is associated with a nearly twice the odds of healing by the 16th week of care. Associations between CEPCSs have also been confirmed in two small cohort studies and now validated in this larger prospective multicenter cohort study. Similar findings were noted for CEPCSs as determined by other cell markers 34^{+}45^{dim}146^{dim}, 34^{+}117^{+}45^{dim}146^{dim}, and 34^{+}117^{+}146^{+}45^{dim}. Based on previous work showing associations with *NOS1AP* variation, our working hypothesis is that circulating capon levels that may be influenced by *NOS1AP* variation is inversely associated with CEPCSs and ultimately wound repair. CEPC number was inversely associated with capon; however, due to technical issues, we had inadequate samples to evaluate an association between circulating capon and a healed wound. MP from various cell sources have been associated with both beneficial and detrimental aspects of angiogenesis and tissue repair, however, MPs as measured in this study do not appear to be associated with DFU healing.

A previous study of 100 individuals with DFU by Thom et al evaluated CEPCs (CD34^{+} CD45^{dim}) and noted that an increase in CEPCs during the first two weeks of care was associated with healing by the 16th week of care. Interestingly, the number of CEPCSs at baseline was greater in the 37% of individuals that healed, but the difference between those that healed and those unhealed was not significant unless adjusted for wound area, patient age and hypoxia inducible factor (HIF).(15) The previous study failed to describe the importance of wound size alone on CEPCS number, which in the current study was the important confounder. The importance of wound area with respect to DFU wound healing is well known and was recently shown to be pivotal with respect to DFUC.(26; 27; 29; 34; 35) The association with wound size is important due to the heterogeneity of DFUs at presentation and the generalizability of observations with respect to wound repair in this and other studies as well as the association of size with severity and failure to heal.(26; 27; 34; 35) Furthermore, in the current study we were able to show that as a prognostic factor, CD34^{+} CD45^{dim}, is by itself a helpful prognostic factor and improves an accepted prognostic model that contains wound area and wound duration (AUC= 0.75(0.67, 0.82)). Finally, log transformed CD34^{+} CD45^{dim} per log transformed initial wound area, as a

solitary predictor, is an excellent predictor with an AUC of 0.72 (0.64, 0.79). Thereby, potentially helping a clinician determine who might early advanced adjuvant care.

The mechanism of action of CEPCs on wound healing is not clear. Previous randomized clinical trials have indirectly tried to manipulate CEPC numbers with mixed results. For example, granulocyte-macrophage colony stimulating factor (GM-CSF) has been shown to increase the number of CEPCSs by increased bone marrow proliferation and extravasation of CEPCSs.(36; 37) However, reports have not consistently shown GM-CSF to improve the likelihood that a wound will heal.(38–41) It is possible based on our report that adjusting GM-CSF dose by wound area could result in more consistent results.

Our study has limitations, in that, it was designed to replicate previous studies regarding CEPCSs, to expand our knowledge about CEPCS sub types, and to explore potential associations between MPs and DFU wound healing. Our study was multicentered and enrolled a diverse group of subjects from different wound care environments. However, it was still not large enough to know if our findings generalize to all patients seen in all wound care environments. Wound care centers followed their standard protocol, which was similar from site to site. It remains to be seen whether using therapies that increase CEPCSs might increase the likelihood that a wound will heal, as our data does not directly provide an answer to that query. We focused only on information obtained at the first visit, without knowledge of future treatment options, so it is unlikely repeated measure of the parameters evaluated could substantially add to our ability to understand the importance of CEPCSs with respect to DFU.

In summary, CEPCs measured at the first encounter in an individual with a DFU, are associated with the likelihood that a wound will heal by the 16^{th} week of care. The association between CEPCs is affected by wound area and wound duration at the first office visit. This study improves the generalizability and validity of a previous study of CEPCS. (15) As compared to the previous study we show that several CEPCSs that vary by the maturity of the endothelial cell are all associated with a wound that heals. The effect of CEPCS on wound healing is not associated with other DM risk factors or comorbidities like renal disease, vascular disease, or duration of diabetes. Based on this study and others, CEPCSs measured at the first office visit is a viable prognostic factor for healing of a DFU. (15) Area adjusted $CD34^{+}$ $CD45^{dim}$ is likely strong predictor of the likelihood of a healed DFU by the 16^{th} week of care. Additional studies are indicated to determine if increasing CEPCSs in patients with DFU might improve the wound healing.

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Table 1:

Patient and wound characteristics at the first study visit by those that healed by the 16th week of care or did not heal. Means or percentages with 95% CI.

Characteristic	N	Unhealed	Healed
Sex (male)%	204	74.24 (66.68,81.80)	70.83 (60.08,81.59)
Age at enrollment	202	57.51 (55.85,59.17)	58.32 (55.99,60.65)
Black%	204	57.58 (49.03,66.12)	59.72 (48.12,71.33)
Age of diabetes onset	180	39.55 (37.06,42.03)	39.28 (36.28,42.28)
ho Peripheral Arterial Disease %	198	24.03 (16.56,31.50)	18.84 (9.38,28.30)
ho Chronic Kidney Disease %	166	30.09 (21.50,38.68)	28.30 (15.77,40.84)
# of Wounds	202	1.39 (1.27,1.52)	1.26 (1.11,1.42)
ln Wound Duration	193	3.08 (2.89,3.27)	2.59 (2.29,2.89) *
ln Wound Area	200	1.39 (1.07,1.71)	0.05 (−0.40,0.50) *
ho Neuropathy %	196	86.51 (80.46,92.56)	92.86 (86.67,99.04)
Wound Depth: Dermis only %	204	30.30 (22.36,38.25)	22.22 (12.38,32.06)
ho previous Amputation %	202	47.69 (38.86,56.63)	48.61 (37.65,60.69)

* No significant differences except wound duration (p=0.012) and wound area (p<0.001)

Table 2:

Natural log of CEPCS per 100,000 WBC, MP as total number of annexin V positive particles < 1 μm per ml and leukocyte NOS1AP protein per actin content for individuals healed or not healed by the 16th week of care and standard deviation.

Circulating marker	Unhealed	Healed
CEPCS-CD34^{+}45^{dim}	3.12(1.04)	3.29(0.94)
CEPCS-CD34^{+}45^{dim}146^{dim}	3.02(0.98)	3.16(0.91)
CEPCS-CD34^{+}117^{+}45^{dim}146^{dim}	1.53(0.85)	1.73(0.88)
CEPCS-CD34^{+}117^{+}146^{+}45^{dim}	−2.32(1.47)	−2.06(1.14)
CEPCS-CD34^{dim}117^{+}90^{+}	1.46(0.93)	1.49(1.00)
CEPCS-CD34^{dim}117^{+}146^{+}	1.04(1.44)	1.18(1.88)
MP-CD146^{+}	7.36(1.46)	7.42(1.32)
MP-CD90^{+}	5.34(1.68)	5.49(1.31)
MP-CD117^{+}146^{+}34^{dim}	6.24(2.23)	6.20(2.39)
MP-CD117^{+}90^{+}34^{dim}	6.09(2.33)	5.84(2.55)
MP-CD34^{+}	5.20(1.58)	4.93(1.49)
MP-Phalloidin^{+}	6.91(1.60)	6.92(1.51)
NOS1AP protein	1.05(1.76)	1.08(2.63)
CEPCS-CD34^{+}45^{dim} per wound area	1.75(1.97)	3.26(1.83)

Table 3:

Odds ratios (OR) for healing comparing those that healed by the 16th week of care to those who did not relative to natural log of CEPCS/100,000 WBC. Adjusted models were adjusted for natural log of wound area and wound duration.

	Univariate		Adjusted	
Circulating marker	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**
CEPCS-CD34^{+}45^{dim}	1.19 (0.88,1.61)	.2536736	1.67 (1.16,2.42)	.0060895
CEPCS-CD34^{+}45^{dim}146^{dim}	1.17 (0.85,1.60)	.346676	1.62 (1.11,2.35)	.012492
CEPCS-CD34^{+}117^{+}45^{dim}146^{dim}	1.32 (0.93,1.88)	.1256584	1.58 (1.07,2.32)	.0219954
CEPCS-CD34^{+}117^{+}146^{+}45^{dim}	1.16 (0.92,1.46)	.2207525	1.46 (1.08,1.98)	.0150963
CEPCS-CD34^{dim}117^{+}90^{+}	1.03 (0.74,1.42)	.8694772	1.20 (0.84,1.72)	.3138301
CEPCS-CD34^{dim}117^{+}146^{+}	1.05 (0.87,1.27)	.5900635	1.25 (1.00,1.56)	.0524155

Table 4:

Odds ratios (OR) for healing comparing those that healed by the 16th week of care to those who did not relative to natural log of MPs. Adjusted models were adjusted for natural log of wound area and wound duration.

	univariate		Adjusted	
Circulating marker	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**
MP-CD146^{+}	1.03 (0.83,1.27)	.7976976	1.05 (0.83,1.33)	.6852645
MP-CD90^{+}	0.98 (0.81,1.19)	.8515515	1.06 (0.86,1.31)	.5972152
MP-CD117^{+}146^{+}34^{dim}	0.99 (0.87,1.14)	.9197814	1.09 (0.93,1.29)	.2870091
MP-CD117^{+}90^{+}34^{dim}	0.96 (0.85,1.08)	.4779338	1.06 (0.92,1.23)	.3999426
MP-CD34^{+}LN	0.89 (0.74,1.08)	.2463308	0.98 (0.80,1.22)	.8809028
MP-Phalloidin^{+}	1.01 (0.84,1.21)	.9393344	0.98 (0.80,1.21)	.8573239