Chapter 6

Angiogenesis and vasculogenesis: Inducing the growth of new blood vessels and wound healing by stimulation of bone marrow–derived progenitor cell mobilization and homing

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During embryonic development, the vasculature is among the first organs to form and is in charge of maintaining metabolic homeostasis by supplying oxygen and nutrients and removing waste products. As one would expect, blood vessels are critical not only for organ growth in the embryo but also for repair of wounded tissue in the adult. An imbalance in angiogenesis (a time-honored term that globally refers to the growth of new blood vessels) contributes to the pathogenesis of numerous malignant, inflammatory, ischemic, infectious, immune, and wound-healing disorders. This review focuses on the central role of the growth of new blood vessels in ischemic and diabetic wound healing and defines the most current nomenclature that describes the neovascularization process in wounds. There are now two well-defined, distinct, yet interrelated processes for the formation of postnatal new blood vessels, angiogenesis, and vasculogenesis. Reviewed are recent new data on vasculogenesis that promise to advance the field of wound healing. (J Vasc Surg 2007;45:39A-47A.)

It is now well established that an essential part of normal healing for full thickness cutaneous wounds is the formation of new blood vessels within the provisional wound matrix that is referred to as granulation tissue. Neovascularization of the wound's granulation tissue occurs by the processes of angiogenesis or vasculogenesis, or both.[1] Angiogenesis refers to the process by which resident endothelial cells of the wound's adjacent mature vascular network proliferate, migrate, and remodel into neovessels that grow into the initially avascular wound tissue aided by mature stromal cells such as fibroblasts.[1-6] Vasculogenesis is a de novo process by which progenitor stem cells differentiate and give rise to a replacement vascular network.[7-9] It was once believed that vasculogenesis only occurred during embryonic life; however, bone marrow-derived endothelial progenitor cells (BMD EPCs) have been identified in peripheral blood in adults and participate in new vessel formation.[7,10]

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BONE MARROW-DERIVED ENDOTHELIAL PROGENITOR CELLS

BMD EPCs contribute to wound healing because these progenitor/stem cells are the key cellular effectors of postnatal vasculogenesis. BMD EPCs given to animals with surgically induced limb ischemia incorporate into foci of neovascularization in ischemic muscle, skin, and wounds.[7-13] We have recently identified a critical role for BMD EPCs in ischemic wound healing.[14] We quantified the contribution of BMD EPCs to wound healing with and without ischemia in chimeric mice formed using bone marrow from FVB/Tie-2-LacZ transgenic mice (FVB/N-TgN[TIE2LacZ]182Sato, The Jackson Laboratory, Bar Harbor, Me). Tie-2-LacZ mice are well suited for specifically tracking BMD PCs of the endothelial cell lineage because the endothelial-specific Tie-2 promoter is linked to the LacZ reporter gene allowing cells to be identified by β-galactosidase (β-gal) expression.

We used a murine model of hind limb ischemia induced by femoral ligation/excision (Fig 1) in the chimeric mice we created (Fig 2, *A*). Hind limb ischemia was monitored using laser Doppler flowmetry that allows for quantifying cutaneous blood flow in the ischemic relative to the nonischemic hind limb. Hind limb ischemia resulted in delayed wound healing (Fig 2, *A-C*). We then compared acutely healing wounds in nonischemic hind limbs with delayed healing wounds in the contralateral ischemic hind limb and correlated healing rates to

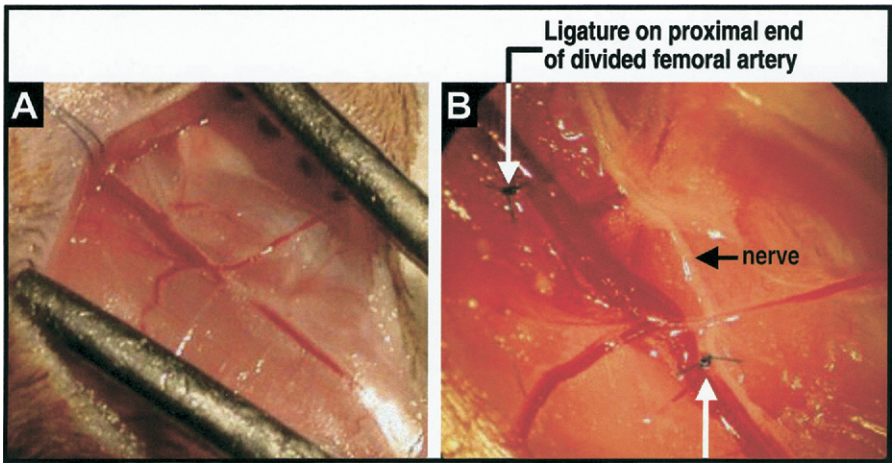

Fig 1. The murine femoral ligation model. **A,** Exposure of femoral neurovascular bundle. **B,** Dissection of the femoral vein and nerve away from the artery with selective femoral artery ligation/excision in FVB mouse.

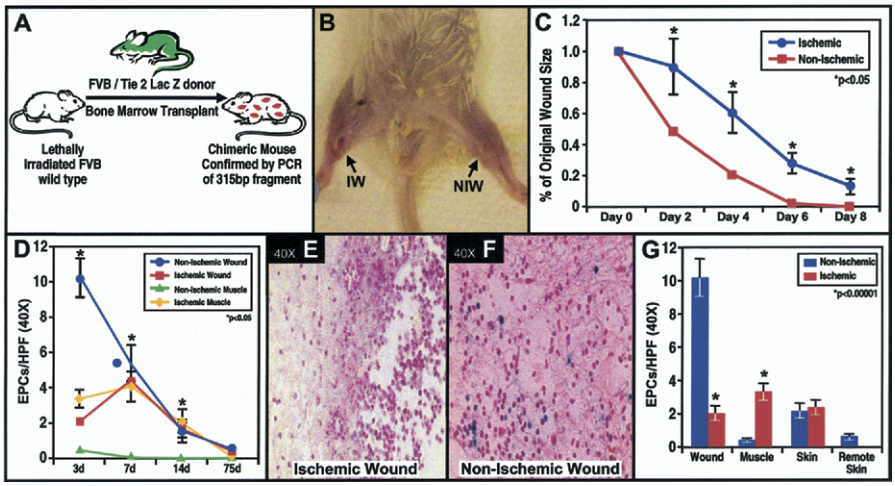

Fig 2. Bone marrow–derived endothelial progenitor cells *(EPC)* recruited to wounds. **A,** The model. **B,** Delay in healing in ischemic wound *(IW)* vs rapid healing in nonischemic wound *(NIW)*. **C,** Wound closure rates (n = 7 per time point) are shown with mean ± standard error in ischemic (triangles) and nonischemic (squares) wounds. *D,* Quantification of EPCs in wounds (ischemic, *diamonds*; nonischemic, *triangle*) and underlying muscle (nonischemic, *squares*; ischemic, *plain*) shows increased EPCs in acutely healing nonischemic wounds at days 3 and 7 (data presented as mean ± standard error). **E, F,** Representative wounds at day 3 (EPCs are β-Gal$^+$ cells seen staining blue). **G,** EPCs per high power fields *(HPF)* in wound, underlying muscle, adjacent skin, and remote skin at day 3 after wounding (data presented as mean ± standard error). (Data and modified figure reprinted with permission from Bauer et al.[14])

BMD EPC recruitment into wounds. We determined that BMD EPCs play a key role in wound healing and are recruited into the granulation tissue of rapidly healing (nonischemic) wounds in significantly greater numbers than into delayed-healing (ischemic) wounds (Fig 2, *D-G*). These studies show that BMD EPCs contribute to acute wound healing, and the process is deficient or incomplete at the level of skin wounds in the presence of severe ischemia.[14]

In diabetic patients and diabetic murine models, the number and function of circulating BMD EPCs is severely impaired, and this defect is highly correlated with the long-term cardiovascular and wound-healing complications seen in diabetes mellitus.[15-19] Increasing evidence suggests that wound-healing mechanisms, in both the bone marrow and within the peripheral wound, are compromised by diabetes as a result of BMD EPC impairments.[15-20] Although cytokines such as granulocyte colony-stimulating factor (GM-CSF) and growth factors such as vascular endothelial growth factor-A (VEGF-A) can induce the release of progenitor cells from the bone marrow, the nonspecific effects on release of other white

cells and platelets or the leaky-capillary effect has made these factors unsuitable to treat diabetic patients with nonhealing chronic wounds.[21-25]

HYPEROXIA AS A SAFE CLINICAL TOOL

Systemic hyperoxia induced by hyperbaric oxygen (HBO_2) is a treatment approved by the United States Food and Drug Administration (FDA) as a safe, adjunctive therapy to stimulate wound healing in diabetic patients. Patients typically receive ≥20 treatments with pure oxygen at 2.0 to 2.4 atmospheres absolute (ATA) once or twice daily. Controlled trials have shown efficacy for HBO_2 in refractory diabetic wounds, but the mechanisms of action are poorly understood.[26-31] HBO_2 is not uniformly effective, particularly in diabetic patients with associated peripheral arterial disease (PAD), accounting for the fact that diabetic/ischemic chronic nonhealing lower extremity wounds continue to be an unsolved clinical problem.

Recent investigations from our laboratory indicate that hyperoxia therapeutically stimulates progenitor/stem cell release from the bone marrow, but these cells may be effectively recruited to wounds to enhance vasculogenesis and healing only if the cytokine milieu in the cutaneous wound bed is optimized.[32-34] Using ischemic and diabetic murine models, we have recently determined that hyperoxia, induced by a clinically relevant HBO_2 protocol, increases nitric oxide (NO) levels within femoral bone marrow, accelerates the spontaneous revascularization of surgically induced hind limb ischemia, and increases the number of BMD PCs in circulation and within cutaneous hind limb ischemic incisional wounds and diabetic excisional wounds.[33,34] These effects appear to be specific to the release of BMD EPC and responsive to the cytokine milieu of the wound.[33,34]

In the ischemic and diabetic murine models that were used, therapeutic wound-healing effects of increased BMD EPC mobilization into circulation and recruitment into wounds were observed in association with enhancement of neovascularization of the wounds and spontaneous recovery of hind limb perfusion.[33,34]

ANGIOGENESIS AND VASCULOGENESIS IN WOUND HEALING

It is increasingly evident that wound healing occurs because of events in two compartments. Within the bone marrow, various signaling pathways lead to mobilization of BMD EPCs and other progenitor/stem cells involved in the healing cascade. Within the wound, neovascularization occurs because of local factors that stimulate adjacent cells (angiogenesis) and because of recruited circulating BMD EPCs that contribute to existing and new vascular channels (vasculogenesis). Vascular maintenance, repair, and wound-healing cellular and molecular cascades at the level of both the bone marrow and within the peripheral wound are compromised by diabetes as a result of BMD EPC impairments.[15-20] The use of cytokines and growth factors such as GM-CSF and VEGF-A to stimulate the bone marrow release of progenitor stem cells for purposes such as wound healing or therapeutic neovascularization has been considered, but generalized application has been thwarted because of risks such as acute arterial thrombosis, angina, hypotension, sepsis, and death.[21-25,35]

Overall, normal cutaneous wound healing proceeds through an orderly sequence of steps that require the control of contamination and infection, resolution of inflammation, regeneration of the connective tissue matrix, angiogenesis/vasculogenesis, wound constriction, and reepithelialization. Chronic wounds are those that have failed to follow this sequence and do not achieve a sustained anatomic and functional result.[36]

The hypoxic nature of all wounds has been demonstrated, but when hypoxia is pathologically increased, wound healing is impaired and the rate of wound infection increases.[35-40] Local oxygen tensions in the vicinity of the wound are approximately half the values observed in normal, unwounded tissue.[41] Fibroblast replication, collagen deposition, angiogenesis, vasculogenesis, and intracellular leukocyte bacterial killing are oxygen-sensitive responses essential to wound healing.[33,34,39,42-44] For these reasons, a number of investigators have examined the role of hyperoxia in wound healing.[26-31,45] This area remains an open field for investigation because, for the most part, chronic wounds are (to this date) a major unsolved clinical problem.

THE UNSOLVED CLINICAL PROBLEM OF LOWER EXTREMITY CHRONIC WOUNDS

It has been estimated that up to 2 million Americans have nonhealing lower extremity wounds that account for 162,500 annual hospitalizations and $1 billion per year in health care costs in the United States.[46-48] Most are the result of diabetes alone or with arterial insufficiency, venous stasis, and neuropathy. These chronic wounds are more common in women, elderly patients, diabetic patients, and African American patients, and result in diminished quality of life, limb loss, and increased morbidity and mortality. In diabetic patients, morbidity due to chronic wounds is staggering, with more than half of all lower limb amputations in the United States occurring in patients with diabetes.

Diabetes is reaching epidemic proportions in Western societies, and is predicted to affect 300 million people worldwide by 2025.[49,50] Nearly 800,000 new cases of diabetes mellitus are diagnosed each year in the United States, and a lower extremity ulcer will develop in approximately 15% of patients at some point in their lives.[51-54]

The pathophysiology of diabetic lower extremity ulcerations and delayed healing has been well described. Contributing factors include progressive development of asensory, vasomotor, and autonomic neuropathy leading

to loss of protective sensation, joint and bone deformities that increase plantar foot pressure, and alterations in autoregulation of dermal blood flow. Diabetic patients show earlier development and progression of lower extremity peripheral arterial occlusive disease (PAD), with a predilection for the trifurcation level of vessels just distal to the knee.

In addition, the tissue microcirculation is severely diseased (microangiopathy), even in patients with patent proximal vessels. Some of these vascular complications in diabetes, as well as the healing defects, have been associated with a decrease in number and function of circulating BMD EPC.[15-20] Impaired host responses to infection and other cellular dysfunctions also contribute to the refractory nature of diabetic wounds. About 20% of diabetic lower extremity ulcers have arterial flow insufficiency as their primary etiology, approximately 50% will have primary diabetic neuropathy, and about 30% will have both conditions.[53,55]

Even after correction of large blood vessel dysfunction by open surgical or endovascular revascularization, only about 47% of patients will heal in a span of 20 weeks with standardized treatment including glycemic control, débridement of necrotic tissue, control of infection, use of moist dressings, protection from pressure or trauma related to ambulation, and adjuvant HBO_2 therapy.[26,56-58]

Hyperbaric oxygen therapy is currently a clinical adjunctive therapy used to stimulate wound healing in situations where the microvasculature has become attenuated but when the large inflow vessels remain open or have been revascularized. Pronounced tissue-level hyperoxia is the main effect of HBO_2 treatments, which are known to raise arterial oxygen tension to several thousand Torr and tissue oxygen tension to about 300 Torr.[59] The effectiveness of HBO_2 as an adjuvant clinical therapy for the treatment of diabetic lower extremity ulcerations has been supported in evaluations by a growing number of studies.[57,60-65] The clinical protocols in current use are not always effective and were arbitrarily determined because fundamental mechanisms are unknown. Thus, it is hoped that ongoing research in this area will yield optimization of the protocols, or reduction of the nonresponder rate, or both.

MOBILIZATION OF STEM CELLS FOR ANGIOGENESIS, VASCULOGENESIS, AND WOUND HEALING

Studies in mice, from our laboratory and others, indicate that the marrow is likely to be a central source for mobilized progenitor/stem cells.[32-34,66,67] Emigration of cells from the bone marrow is generally thought to occur after a period of cell proliferation within the marrow niche.[68-70] In addition, the reported evidence also indicates a rapid mobilization of stem cells, which suggests that cell proliferation is not always necessary. In mice, infusion of soluble Kit ligand (the soluble portion of the c-Kit receptor) triggers mobilization of $CD34^+$ cells in 1 hour.[71] There was a fourfold elevation in circulating progenitor cells within 10 minutes when human volunteers were subjected to highly strenuous exercise.[72]

Data also indicate that specialized microenvironments exists in the marrow where stem/progenitor cells exhibit different propensities for proliferation and mobilization and from where matrix metalloproteinase-9 (MMP-9) activity mediates BMD EPC release.[73-75] From our findings[32-34] and reports by Nakamura et al[71] and Rehman et al,[72] it appears a subpopulation of BMD EPCs exist within specialized bone marrow niches that are poised for rapid release to the circulation.

Nitric oxide has been shown to play a central role in the bone marrow mobilization and release of EPC.[76] Because hyperoxia, induced by HBO_2 increases nitric oxide synthase (NOS) activity in cerebral cortex tissue, perivascular pulmonary tissue, and neutrophils,[77-79] we began to study whether HBO_2 may initiate stem/progenitor cell release by NO-mediated mechanisms. We recently reported that in mice and patients, HBO_2 increases the number of circulating progenitor stem cells and BMD EPC[32,33] (Figs 3, 4, and 5).

Mice were exposed to sham pressurization (placed in the hyperbaric chamber flushed with air at ambient pressure) or to O_2 at 1 ATA or 2.4 ATA for 90 minutes. There was no deviation in circulating cells observed in mice exposed to sham pressurization or 1 ATA O_2, but significant effects occurred in those exposed to 2.4 ATA O_2 (Fig 3).[32,33] Scatter dot-plots and histograms (Fig 3, *A*) exhibit the elevation in the $CD34^+$ and stem cell antigen-1^+ (Sca-1^+) cell populations in a representative control mouse, at 16 hours after exposure to 2.4 ATA O_2, and in a mouse pretreated with NG-nitro-L-arginine methyl ester (L-NAME), a nonspecific NOS inhibitor before HBO_2 (Fig 3, *B* shows the quantification in 22 mice). These data indicate that HBO_2 stimulates stem cell release from the mouse bone marrow by a NO-mediated mechanism. This hyperoxia-induced mobilization of progenitor/stem cells from the bone marrow is highly specific to the release of BMD EPCs and is associated with a therapeutic vasculogenesis and wound-healing response.[33]

The mobilized primitive cells appear to be functional. The HBO_2-mediated elevation in circulating Sca-1/$CD34^+$ cells results in an increase in the colony-forming cell capacity of circulating cells. As shown in Fig 4, there is a significant elevation in the colony-forming cell capacity after exposure to HBO_2 that is inhibited by pretreatment with L-NAME. Again, these data strongly suggests that hyperoxia augments mobilization of functional stem/progenitor cells into the circulation by a NO-mediated mechanism. In fact, direct, real time measurements in fluctuations of bone marrow NO levels show that bone marrow NO level drastically rises in response to hyperoxia, an effect completely inhibited by L-NAME.[33]

The number of circulating stem/progenitor cells expressing the progenitor cell CD34 epitope is also clearly increased by HBO_2 in human subjects[32] (Fig 5). The expression of the

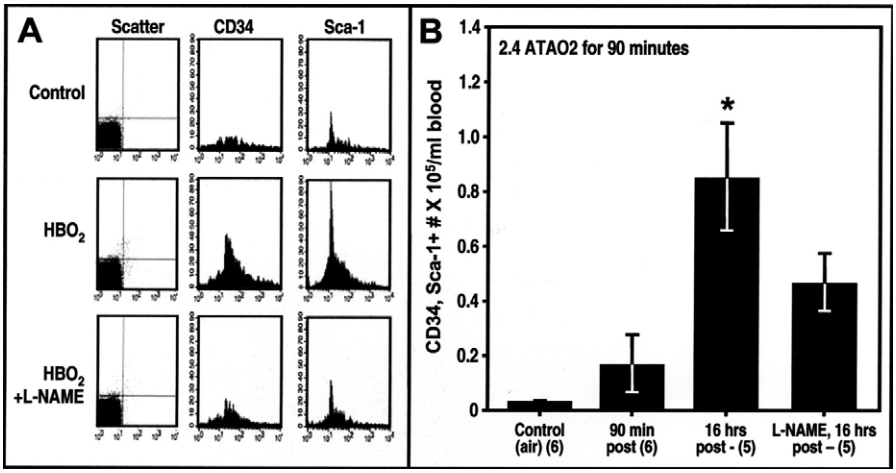

Fig 3. A, Scatter-plots and histograms for stem cell antigen-1 *(Sca-1)*/CD34-expressing cells in peripheral blood from an air-exposed, control mouse; a mouse sacrificed 16 hours after exposure to 2.4 ATA O_2 for 90 minutes, and a mouse first injected with the nonspecific nitric oxide synthase inhibitor, NG-nitro-L-arginine methyl ester *(L-NAME*; 40 mg/kg), 30 minutes before exposure to hyperbaric oxygen *(HBO_2)* and killed 16 hours after exposure. Histograms exhibit cells from three quadrants of the scatter diagrams, excluding only those cells in the lower left quadrant. Plots were gated for small lymphocytes and scans were obtained by counting 50,000 gated events. **B,** Mean elevations of $CD34^+$ and $Sca\text{-}1^+$ cells in peripheral circulation of mice. Data are mean ± standard error for control mice, immediately after exposure to HBO_2 at 2.4 ATA for 90 minutes, at 16 hours post-HBO_2, and in mice first injected with L-NAME before HBO_2. The number of mice included in each group are indicated by (n) in the columns, $*P < .05$ (analysis of variance). Data and modified figure reprinted with permission fromThom et al.[32] *RPE,* R-phycoerythrinis; *FITC,* Fluorescein isothiocyanate conjugated.

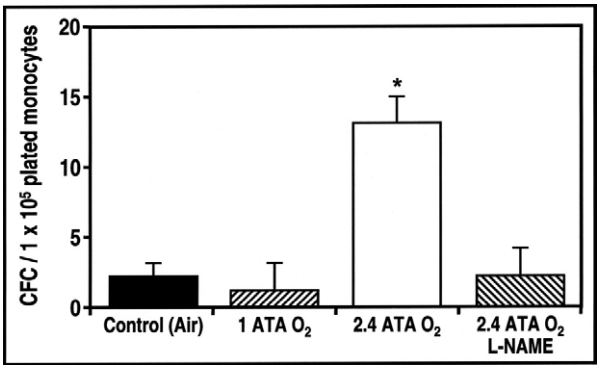

Fig 4. Colony-forming cell *(CFC)* capacity of monocytes from blood with or without hyperbaric oxygen treatment as designated. Mice were sacrificed 16 hours after hyperbaric oxygen exposure for 90 minutes (n =5 mice/group. $*P < .05$ (analysis of variance. *L-NAME;* NG-nitro-L-arginine methyl ester. (Data and modified figure reprinted with permission from Thom et al.[32])

cell-surface antigen CD34 is restricted to primitive progenitor/stem cells of hemangioblast lineage, which give rise to hematopoietic and endothelial progenitors.[80] There was a significant increase in $CD14/CD34^+$ cells after HBO_2 in all five patients evaluated in our pilot studies.[32] Fig 5 shows data from one representative patient before and after the first and 10th hyperoxia treatments. The number of dually positive cells (CD14/CD34) remained elevated in all patients before and after the 10th and 20th treatments.[32] These initial human studies show that a population of monocytes in peripheral blood coexpressing the hemangioblast progenitor/stem cell marker CD34 and the myeloid marker CD14 is increased in response to HBO_2.[32]

Moreover, using transgenic mice and a bone marrow transplantation model that allows us to specifically track BMD EPC,[33] we showed that these hyperoxia effects on progenitor/stem cell release are highly specific to mobilization of BMD EPC. The increased systemic release of BMD EPC into circulation improves neovascularization and wound healing in murine ischemic excisional hind limb wounds.[33] Hyperoxia, induced by HBO_2 daily treatments (2.4 ATA O_2 for 90 minutes) improves hind limb perfusion by laser Doppler flowmetry after femoral ligation/excision, in mice, and by day 8 after wounding, the ischemic excisional punch biopsy wounds treated daily with HBO_2 heal significantly faster.[33] These effects on BMD EPC mobilization, vasculogenesis, and wound healing were not observed in mice that received treatment with L-NAME before HBO_2, indicating that the improvements in BMD mobilization, vasculogenesis, and wound healing are mediated by NO.

Mobilized progenitor cells can differentiate in situ to form capillary networks.[7,81] Many chemokines and cytokines trigger stem/progenitor cell release by induction of MMP-9 in bone marrow, and NO is clearly linked to these processes.[73-76] Using VEGF-A as a proximal stimulus, Aicher et al[76,82] demonstrated that endothelial NOS becomes activated in bone marrow stroma, NO then S-nitrosylates by paracrine mechanisms and acti-

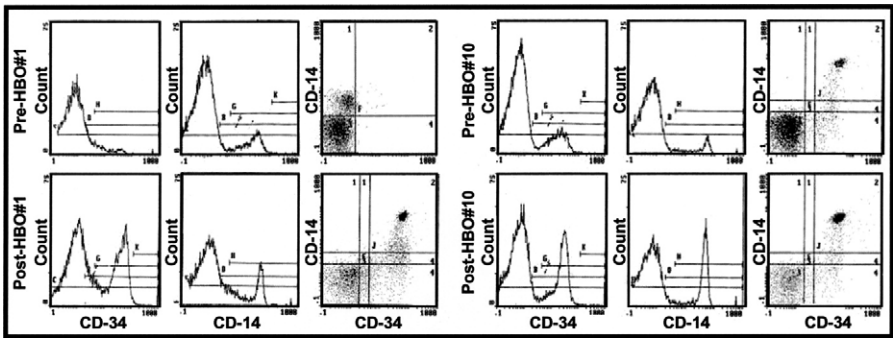

Fig 5. Flow cytometry data from one patient before and after the first and 10th hyperbaric oxygen (*HBO_2*) treatment. Similar results were observed in other patients and in ischemic and diabetic murine models.[32-34]

vates MMP-9, which releases the stem cell active cytokine, soluble Kit ligand. This agent shifts endothelial progenitor and hematopoietic stem cells from a quiescent to the proliferative niche and stimulates rapid stem cell mobilization to the peripheral blood.[12,71,73-76,82] It has been demonstrated that in the setting of trauma and ischemia, systemic VEGF-A levels rise following a time course that mirrors the rise in circulating BMD EPC. Until recently,[33] it was unknown if hyperoxia (acting through similar mechanisms as hypoxia/ischemia or VEGF-A; that is, acting by NO synthesis), can modulate these same angiogenic and vasculogenic signal cascades.

HOMING OF BONE MARROW–DERIVED ENDOTHELIAL PROGENITOR CELLS TO PERIPHERAL CUTANEOUS WOUNDS

The chemokine SDF-1α mediates migration and homing of stem cells, is up-regulated by tissue hypoxia in response to VEGF-A, and serves as the key homing signal guiding BMD EPCs into areas of ischemia (ie, hypoxic tissue).[37,83,84] In healing cutaneous wounds, SDF-1α may function as a homeostatic regulator of tissue remodeling.[85] Its role in promoting wound healing in chronic skin wounds (eg, wounds in diabetic patients or PAD) remains mostly unknown. Most recently, we studied this topic in the streptozotocin diabetic murine model.[34]

Streptozotocin treatment renders mice chemically hypoinsulinemic by causing ablation of the pancreatic Islets of Langerhans, and they can be used to study delayed wound healing in a type 1 diabetes model.[86] The wound-healing impairment can be furthered exacerbated by ischemia induced by surgical femoral artery excision.[1,14] SDF-1α mediates BMD EPC homing through its receptor CXCR4.[37] At the unwounded tissue level, EPC recruitment depends on ischemia-induced or hypoxia-induced upregulation of SDF-1α.[37] Until recently, the role of SDF-1α in diabetes-related delayed healing and in PAD remained mostly unknown. We analyzed the cutaneous wounds of streptozotocin-diabetic mice and were intrigued to determine that the baseline levels of cells staining positive for SDF-1α are significantly decreased in the diabetic wounds.[34] Not surprisingly, the streptozotocin-diabetic mice showed decreased cutaneous wound closure rates.[34]

Our data indicate that the diabetic impairments in BMD EPC recruitment can be therapeutically addressed by improving the cytokine milieu of the wound and could potentially be further synergistically impacted by a systemic therapy (eg, hyperoxia) that aims at increasing the circulating pool of BMD EPCs.

The data also indicate that hyperoxia increases wound closure rates in the ischemic and the diabetic murine models, likely by significantly augmenting the circulating pool of BMD EPCs and impacting both angiogenesis and vasculogenesis. NOS inhibition blocks these hyperoxia-induced therapeutic effects.[33,34] Hyperoxia alone does not have a significant impact on BMD EPC homing to wounds with delayed healing.

On the basis of these recent findings, it is our current working hypothesis that hyperoxia induces the release of BMD EPCs into circulation by NO mechanisms (eNOS/NO) and that these crucial reparative and vasculogenic cells may then be recruited into wounds in increased numbers by virtue of their hyperoxia-induced activation and their increased numbers within the blood pool; however, local wound interventions that enhance EPC homing (such as increasing level of EPC homing chemochine, SDF-1α) may be crucial for optimal therapeutic recruitment of these progenitor cells to diabetic and ischemic wounds.[34]

CONCLUSIONS

BMD EPCs are the newest cellular target that may be used to influence postnatal angiogenesis and vasculogenesis. Recent data demonstrate that intermittent systemic hyperoxia induced by FDA-approved HBO_2 protocols may be a currently available, safe, and clinically useful tool to enhance BMD EPC mobilization into circulation with minimal if any side effects. Other approaches for systemic progenitor/stem cell mobilization that involve the systemic use of cytokines or growth hormones may carry significant associated side effects related to intra-arterial thromboses (GM-CSF) or leaky capillary syn-

drome (VEGF-A). Whether BMD EPC released into circulation by hyperoxia can effectively be recruited to delayed-healing cutaneous wounds (affected by diabetes or ischemia, or both), or whether local wound treatments such as SDF-1α may achieve synergism with hyperoxia in regards to the BMD EPC recruitment to compromised cutaneous wounds in diabetic patients, will require further study.

We have determined that hyperoxia-mobilized progenitor cells may be effectively recruited into murine diabetic or ischemic wounds to enhance wound angiogenesis, vasculogenesis, and healing. Because there is no uniformly effective method to achieve healing in wounds compromised by diabetes and ischemia, and because these chronic wounds represents such a profound, unsolved, and rapidly increasing health problem with exuberant morbidity, mortality, and cost, we believe that this area of research may continue to be fruitful. For example, the delineation of the signals that mediate the hyperoxia-induced progenitor cell release from the bone marrow (upstream and downstream from activation of NOS/NO) will constitute a leap forward in our basic understanding of the biology of BMD EPC mobilization. This latter knowledge may become a strong foundation for further novel targets of therapeutic intervention.

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