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Angiogenesis & 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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Abstract

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. In this review, we will focus on the central role of the growth of new blood vessels in ischemic and diabetic wound healing. We define 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 post-natal new blood vessels, angiogenesis and vasculogenesis. We review recent new data on vasculogenesis that promises to advance the field of wound healing.

Introduction

It is now well established that, for full thickness cutaneous wounds, an essential part of normal healing 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 and/or vasculogenesis.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 EPC) have been identified in peripheral blood in adults and participate in new vessel formation.^{7,10}

BMD EPC

BMD EPC contribute to wound healing since these progenitor/stem cells are the key cellular effectors of post-natal vasculogenesis. BMD EPC given to animals with surgically induced

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limb ischemia incorporate into foci of neovascularization in ischemic muscle, skin, and wounds.^{7–13} We have recently identified a critical role for BMD EPC in ischemic wound healing.¹⁴ We quantified the contribution of bone marrow-derived endothelial progenitor cells 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, Maine). Tie-2-LacZ mice are well suited for specifically tracking bone marrow-derived progenitor cells 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. After creating the chimeric mice (as depicted in Fig. 2A), we used a murine model of hind limb ischemia induced by femoral ligation/excision (as depicted in Fig. 1). Hindlimb ischemia was monitored using laser doppler flowmetry (LDF) that allows for quantifying cutaneous blood flow in the ischemic relative to the nonischemic hind limb. Hindlimb ischemia resulted in delayed wound healing (Fig. 2A–C). We then compared acutely healing wounds in non-ischemic hind limbs with delayed healing wounds in the contralateral ischemic hindlimb and correlated healing rates to BMD EPC recruitment into wounds. We determined that BMD EPC play a key role in wound healing and are recruited into the granulation tissue of acutely healing (non-ischemic) wounds in significantly greater numbers than to delayed-healing (ischemic) wounds (Fig. 2D–G). These studies show that BMD EPC 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 EPC are severely impaired and this defect is highly correlated with the long-term cardiovascular and wound healing complications seen in Diabetes.^{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} While cytokines (e.g. Granulocyte Colony-stimulating Factor (GM-CSF) and growth factors (e.g. Vascular Endothelial Growth Factor-A (VEGF-A)) can induce the release of progenitor cells from the bone marrow, the non-specific effects on release of other white cells and platelets or the leaky-capillary effect has made these factors unsuitable to treat diabetic patients with non-healing chronic wounds.^{21–25}

Hyperoxia as a Safe Clinical Tool

Systemic hyperoxia induced by hyperbaric oxygen (HBO₂) treatments is used as a safe, FDA-approved, adjunctive therapy to stimulate wound healing in diabetic patients. Patients typically receive 20 or more treatments with pure O₂ at 2.0 to 2.4 atmospheres absolute (ATA) once or twice daily. Controlled trials have shown efficacy for HBO₂ in refractory diabetic wounds, but the mechanisms of action are poorly understood.^{26–31} HBO₂ is not uniformly effective, particularly in diabetic patients with associated peripheral arterial disease (PAD), accounting for the fact that diabetic/ischemic chronic non-healing 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 HBO₂ protocol, increases nitric oxide (\cdot NO) levels within femoral bone marrow, accelerates the spontaneous revascularization of surgically induced hindlimb ischemia, and increases the number of bone marrow-derived progenitor cells in circulation and within cutaneous hindlimb 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} Our studies indicate that Stromal cell-derived growth factor-1 α (SDF-1), a chemokine that mediates EPC homing via its receptor CXCR4 (chemokine receptor 4), is decreased in diabetic

excisional wounds and that SDF-1 wound-injections partially restores the diabetic defect in wound BMD EPC recruitment, and (together with hyperoxia) synergistically enhances diabetic wound healing.³⁴ In the utilized ischemic and diabetic murine models, 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 hindlimb 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 EPC 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 EPC 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 due to 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 re-epithelialization. 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, non-wounded 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, by 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 suffer from non-healing lower extremity wounds, accounting for 162, 500 annual hospitalizations and one billion dollars per year in health care costs in the United States.^{46–48} The majority of these wounds are the result of diabetes alone or in combination with arterial insufficiency, venous stasis, and neuropathy. These chronic wounds are more common in women, the elderly, 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 US 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 per year in the United States, and approximately 15 % of patients will develop a lower extremity ulcer 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 sensory, 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. Diabetics 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 as well as the healing defects, in diabetes, 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 (open surgical or endovascular revascularization), only about 47% of patients will heal in a span of 20 weeks with standardized treatment including glycemic control, debridement of necrotic tissue, control of infection, use of moist dressings, protection from pressure or trauma related to ambulation, and adjuvant HBO₂ 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 HBO₂ treatments, which are known to raise arterial O₂ tension to several thousand Torr and tissue O₂ tension to ~ 300 Torr.⁵⁹ The effectiveness of HBO₂ 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, ongoing research in this area is hoped to yield optimization of the protocols and/or reduction of the non-responder rate.

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 CD 34+ cells in 1 hour.⁷¹ There is a four-fold elevation in circulating progenitor cells within 10 minutes when human volunteers were subjected to highly strenuous exercise.⁷² Data also indicate that specialized microenvironments exist in the marrow where stem/progenitor cells exhibit different propensities for proliferation and mobilization and from where MMP-9 activity mediates BMD EPC release.^{73–75} Based on our findings^{32–34} and reports by Nakamura, et. al.⁷¹ and Rehman, et. al.,⁷² it appears that there is a sub-population of BMD EPC within specialized bone marrow niches that is poised for rapid release to the circulation.

·NO has been shown to play a central role in the bone marrow mobilization and release of EPC.⁷⁶ Because hyperoxia, induced by HBO₂ increases nitric oxide synthase (NOS) activity in cerebral cortex tissue, perivascular pulmonary tissue and neutrophils,^{77–79} we began to study whether HBO₂ may initiate stem/progenitor cell release by ·NO-mediated mechanisms. We recently reported that in mice (and patients), HBO₂ increases the number of circulating progenitor stem cells and BMD EPC^{32,33} (Fig. 3–5). Mice were exposed to a sham pressurization (placed in the hyperbaric chamber flushed with air at ambient pressure), to 1 ATA or to 2.4 ATA O₂ for 90 minutes. There was no deviation in circulating cells observed in mice exposed to sham pressurization or 1 ATA O₂, but significant effects occurred in those exposed to 2.4 ATA O₂ (Fig. 3).^{32,33} Scatter dot-plots and histograms (in Fig. 3A) exhibit the

elevation in the CD34⁺ and Stem cell antigen-1 (Sca-1)⁺ cell populations in a representative control mouse, at 16 hours following exposure to 2.4 ATA O₂ and in a mouse pre-treated with L-nitroarginine methyl ester (L-NAME), a non-specific nitric oxide synthase inhibitor prior to HBO₂ (Fig. 3B shows the quantification in 22 mice). These data indicate that HBO₂ 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 EPC and associated with a therapeutic vasculogenesis, and wound healing response.³³ The mobilized primitive cells appear to be functional. The HBO₂-mediated elevation in circulating Sca-1/CD34⁺ cells results in an increase of colony forming cell (CFC) capacity of circulating cells. As shown in Fig. 4, there is a significant elevation in CFC capacity following exposure to HBO₂ that is inhibited by pre-treatment with L-NAME. Again, this 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 nitric oxide 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 HBO₂ in human subjects,³² (Fig. 5). CD34 is a cell-surface antigen whose expression is restricted to primitive progenitor/stem cells of hemangioblast lineage (that give rise to hematopoietic and endothelial progenitors).⁸⁰ There was a significant increase in CD14/CD34 positive cells after HBO₂ in all five patients evaluated in our pilot studies.³² Fig. 5 shows data from one representative patient before and after the 1st and tenth 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 co-expressing the hemangioblast progenitor/stem cell marker CD34, and the myeloid marker CD14, is increased in response to HBO₂.³²

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 hindlimb wounds.³³ Hyperoxia, induced by HBO₂ daily treatments (2.4 ATA O₂ for 90 min) improves hindlimb perfusion by LDF after femoral ligation/excision, in mice, and by day 8 after wounding the ischemic excisional punch biopsy wounds treated daily with HBO₂ 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 prior to HBO₂, 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 chemo/cytokines trigger stem/progenitor cell release via induction of metalloproteinase-9 (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 nitric oxide synthase becomes activated in bone marrow stroma, ·NO then S-nitrosylates (by paracrine mechanisms) and activates 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 via ·NO synthesis), can modulate these same angiogenic and vasculogenic signal cascades.

Homing of BMD EPC to Peripheral Cutaneous Wounds

Stromal-derived factor-1alpha is a chemokine that 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 EPC into areas of ischemia (i.e. hypoxic tissue).^{37,83,84} In healing cutaneous wounds, SDF-1 may function as a homeostatic regulator of tissue remodeling.⁸⁵ SDF-1's role in promoting wound healing in chronic skin wounds (e.g. wounds in diabetic patients or PAD) remains mostly unknown. Most recently, we studied this topic in the Streptozotocin diabetic murine model.³⁴

Streptozotocin (STZ) -treated mice are chemically rendered hypoinsulinemic by STZ-ablation of the pancreatic Islets of Langerhans and 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-1 mediates BMD EPC homing via its receptor CXCR4.³⁷ At the unwounded tissue level, EPC recruitment depends on ischemia-induced or hypoxia-induced upregulation of SDF-1.³⁷ Until recently, the role of SDF-1 in diabetes-related delayed healing and/or PAD remained mostly unknown. We analyzed the cutaneous wounds of STZ- diabetic mice and were intrigued to determine that the baseline levels of cells staining positive for SDF-1 are significantly decreased in the diabetic wounds.³⁴ Not surprisingly, the STZ-diabetic mice showed decreased cutaneous wound closure rates.³⁴ This healing data in the streptozotocin delayed wound-healing diabetic murine model correlates closely with prior reports from other groups. Using this same model, we further observed that compared to the non-diabetic controls, the STZ-diabetic mice show decreased circulating EPC that can be corrected (and augmented) by hyperoxia via a ·NO - mediated mechanism.³⁴ Most significantly, a decreased number of EPC was observed within wound granulation tissue in the STZ-diabetic mice, and this was partially reversed by local wound injections of SDF-1 (25 µg/Kg).³⁴

In these diabetic mice, both systemic hyperoxia treatments and SDF-1 local wound injections, individually increased BMD EPC mobilization and wound closure rates, but only SDF-1 promoted BMD EPC homing to cutaneous wounds.³⁴ The combination of both treatments, synergistically increased BMD EPC mobilization and further significantly enhanced EPC homing to wounds, angiogenesis, vasculogenesis, and wound closure. A significant increase in vasculogenesis and angiogenesis preceded the hyperoxia-induced wound healing effects.³⁴ These 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 (e.g. hyperoxia) that aims at increasing the circulating pool of BMD EPC.

Overall, our findings in murine models confirm that ischemia and diabetes delay wound healing.^{14,33,34} The most recent work³⁴ further indicated that diabetes decreases wound expression of SDF-1, decreases circulating BMD EPC, and decreases wound BMD EPC (the latter being reversed by local wound treatment with SDF-1 injections).³⁴ The data also indicates that hyperoxia increases wound closure rates in the ischemic and the diabetic murine models, likely by significantly augmenting the circulating pool of BMD EPC 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. Based on these recent findings, it is our current working hypothesis that hyperoxia induces release of BMD EPC into circulation via nitric oxide 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 chemokine,

SDF-1) may be crucial for optimal therapeutic recruitment of these progenitor cells to diabetic and/or ischemic wounds.³⁴

Conclusions

BMD EPC are the newest cellular target that may be utilized to influence postnatal angiogenesis and vasculogenesis. Recent data demonstrate that intermittent systemic hyperoxia induced by FDA-approved HBO₂ 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 involves the systemic use of cytokines or growth hormones may carry significant associated side effects related to intra-arterial thromboses (GM-CSF) or leaky capillary syndrome (VEGF-A). Whether BMD EPC released into circulation by hyperoxia can effectively be recruited to delayed-healing cutaneous wounds (affected by diabetes and/or ischemia) or whether local wound treatments such as SDF-1 α 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 such as to enhance wound angiogenesis, vasculogenesis and healing. Since there is no uniformly effective method to achieve healing in wounds compromised by diabetes and ischemia and since 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 nitric oxide synthase/nitric oxide) 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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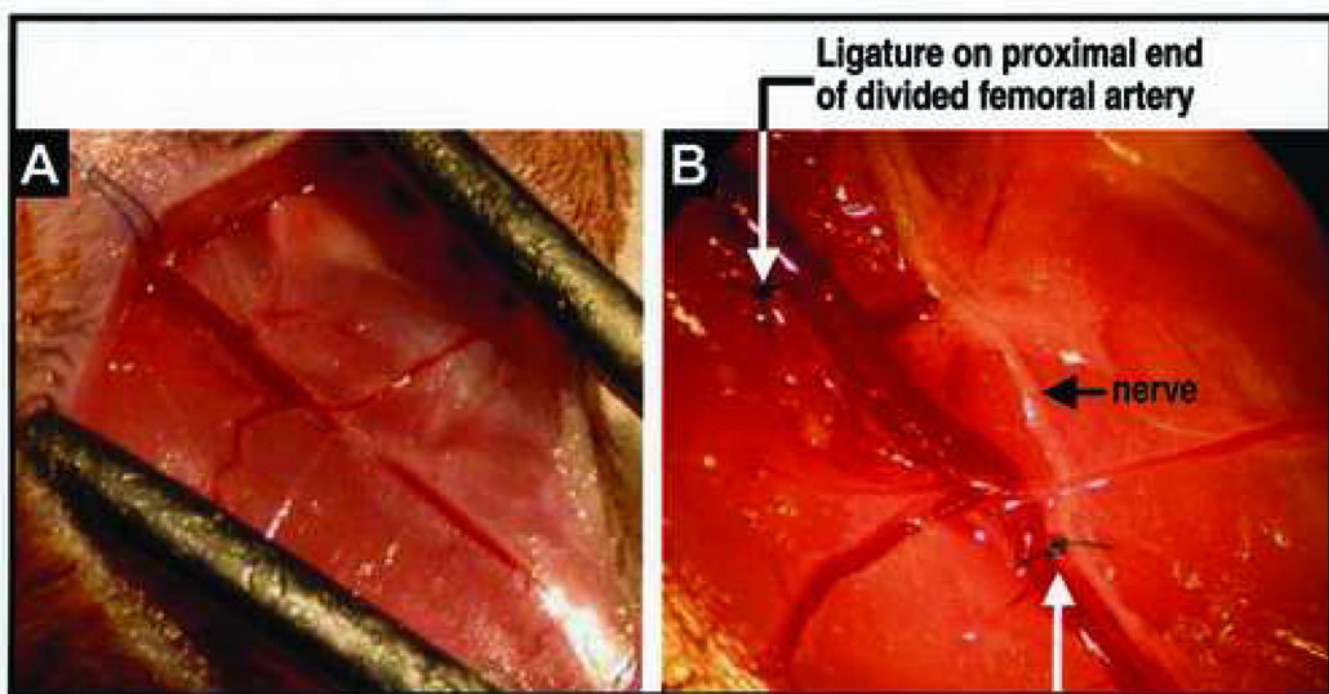


Fig. 1. The murine femoral ligation model. (A) Exposure of femoral neurovascular bundle and (B) Dissection of the femoral vein and nerve away from the artery with selective femoral artery ligation/excision in FVB mouse.

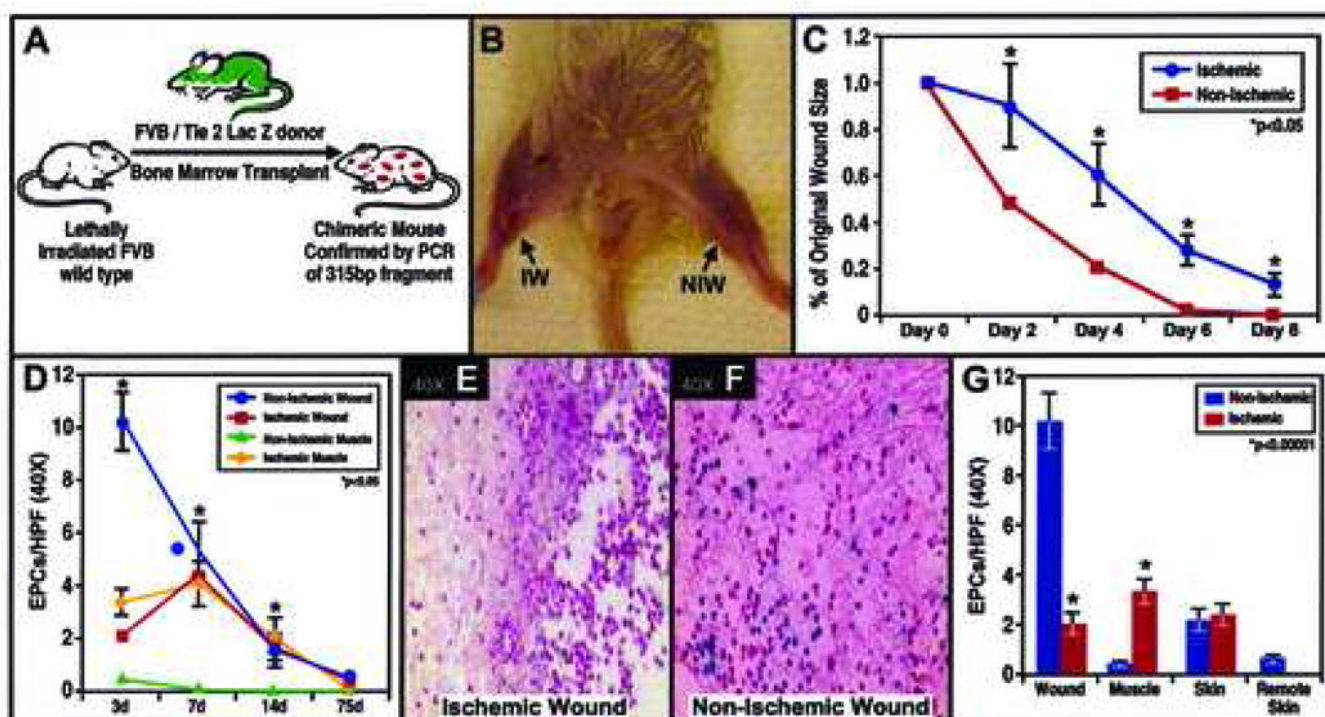


Fig. 2. BMD EPC recruited to wounds. (A). The Model; (B). Delay healing in ischemic wound (IW) vs. Acute healing in non-ischemic wound (NIW); (C). Wound closure rates (N=7 per time point); (D). Quantification of EPC in wounds and underlying muscle showing increased EPC in acutely healing non-ischemic wounds at days 3 and 7; (E-F). Representative wounds at day 3 (EPC are β -Gal + cells seen staining blue); (G) EPC in wound, underlying muscle, adjacent skin, and remote skin at day 3 after wounding. Data and modified figure re-printed with permission from citation No. 14 (Bauer et. al. J Vasc Surg 2006).

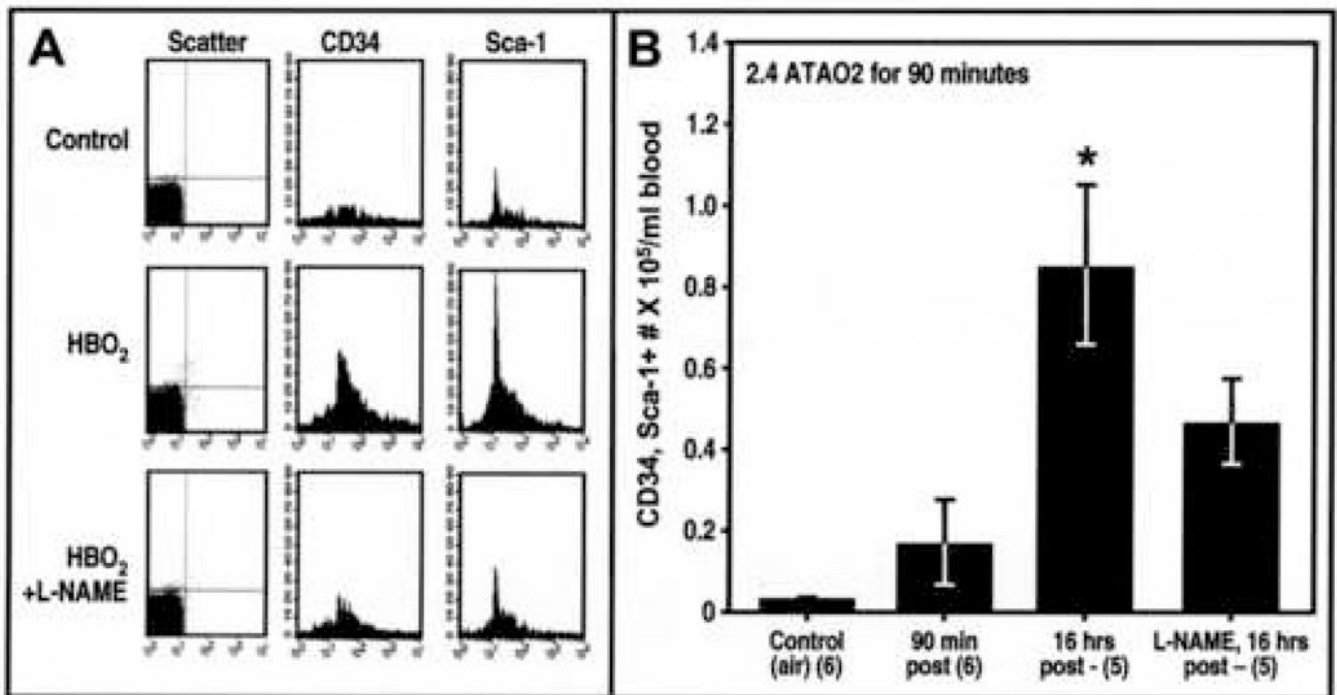


Fig. 3.

(A) Scatter-plots and histograms for 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 O₂ for 90 minutes, and a mouse first injected with the non-specific NOS inhibitor, L-NAME (40 mg/kg), 30 minutes prior to exposure to HBO₂ 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 obtained by counting 50,000 gated events. (B) Mean elevations of CD34+, Sca-1+ cells in peripheral circulation of mice. Data are mean \pm SE for control mice, immediately after exposure to HBO₂ at 2.4 ATA for 90 minutes, at 16 hours post-HBO₂ and in mice first injected with L-NAME prior to HBO₂. The number of mice included in each group are indicated by (n) in the columns, * $p < 0.05$ (ANOVA). Data and modified figure re-printed with permission from citation No. 32 (Thom, et. al. Am J Physiol Heart Circ Physiol 2006).

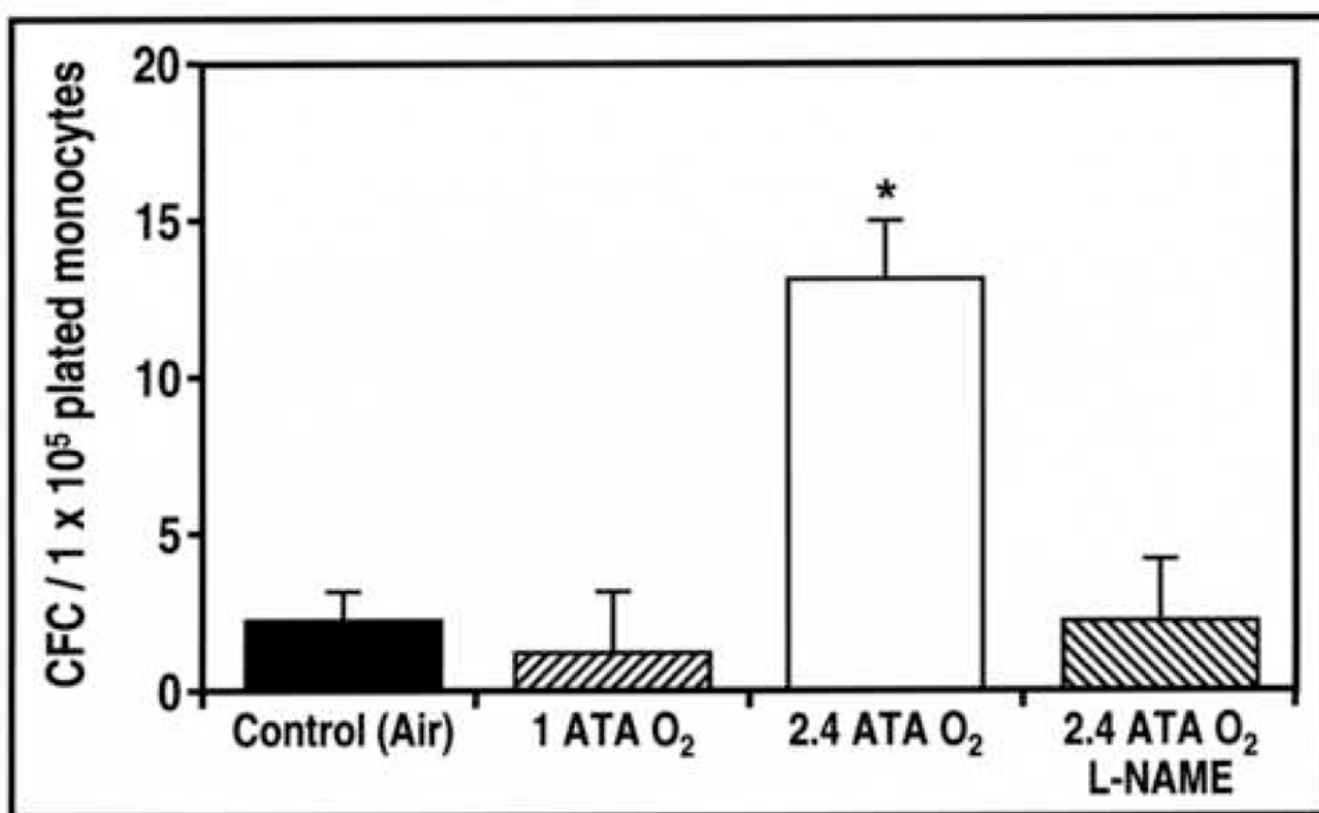


Fig. 4. Colony forming cell (CFC) capacity of monocytes from blood with or without HBO₂ treatment as designated. Mice were sacrificed 16 hr after HBO₂ exposure for 90min. n=5 mice/group, *p<0.05 (ANOVA). Data and modified figure re-printed with permission from citation No. 32 (Thom, et. al. Am J Physiol Heart Circ Physiol 2006).

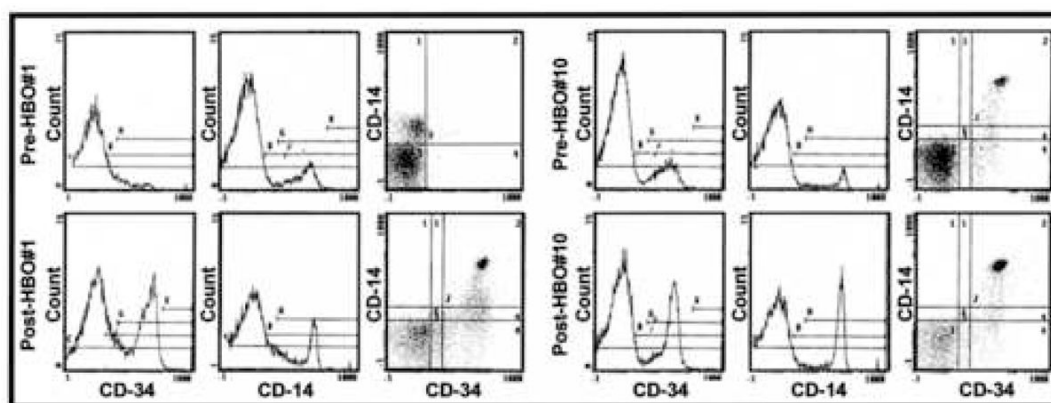


Fig. 5. Flow cytometry data from one patient before and after the 1st and 10th HBO₂ treatment. Similar results were observed in other patients and in ischemic and diabetic murine models.^{32–34}