Hyperoxia and angiogenesis

HARRIET W. HOPF, MD[a,b]; JEFFREY J. GIBSON, MD[a]; ADAM P. ANGELES, MD[a]; JAMES S. CONSTANT, MD[a]; JOHN J. FENG, MD[a]; MARK D. ROLLINS, MD[b]; M. ZAMIRUL HUSSAIN, PhD[a]; THOMAS K. HUNT, MD[a]

We hypothesized that tissue hyperoxia would enhance and hypoxia inhibit neovascularization in a wound model. Therefore, we used female Swiss-Webster mice to examine the influence of differential oxygen treatment on angiogenesis. One milliliter plugs of Matrigel®, a mixture of matrix proteins that supports but does not itself elicit angiogenesis, were injected subcutaneously into the mice. Matrigel® was used without additive or with added vascular endothelial growth factor (VEGF) or anti-VEGF antibody. Animals were maintained in hypoxic, normoxic, or one of four hyperoxic environments: hypoxia—13 percent oxygen at 1 atmosphere absolute (ATA); normoxia—21 percent oxygen at 1 ATA; hyperoxia—(groups a–d) 100 percent oxygen for 90 minutes twice daily at the following pressures: Group a, 1 ATA; Group b, 2 ATA; Group c, 2.5 ATA; Group d, 3.0 ATA. Subcutaneous oxygen tension was measured in all groups. The Matrigel® was removed 7 days after implantation. Sections were graded microscopically for the extent of neovascularization. Angiogenesis was significantly greater in all hyperoxic groups and significantly less in the hypoxic group compared with room air-exposed controls. Anti-VEGF antibody abrogated the angiogenic effect of both VEGF and increased oxygen tension. We conclude that angiogenesis is proportional to ambient pO_2 over a wide range. This confirms the clinical impression that angiogenesis requires oxygen. Intermittent oxygen exposure can satisfy the need for oxygen in ischemic tissue. **(WOUND REP REG 2005;13:558–564)**

Angiogenesis is critical to normal growth, tumor growth, and wound healing.[1–3] As presently understood, it involves at least nine sequential activities, which include endothelial cell activation, lysis of matrix, endothelial cell migration, cell division, tube formation, tube maturation, matrix synthesis, establishment of circulation, and recruitment of pericytes. The current consensus is that wound macrophages produce cytokines and/or growth factors that are the main stimulants for angiogenesis.[4,5] Vascular endothelial growth factor (VEGF) is generally considered a dominant stimulant in wounds.[6,7]

From the Wound Healing Research Laboratory[a], Department of Surgery, and the Department of Anesthesia and Perioperative Care[b], University of California, San Francisco, California.

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Reprint requests: Harriet W. Hopf, MD, Department of Anesthesia and Perioperative Care, Box 0648, Rm. U286, 521 Parnassus Avenue, San Francisco, CA 94143-0648. Fax: (415) 476-1623. Email: hopfh@anesthesia.ucsf.edu.

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ATA	Atmosphere absolute
$PsqO_2$	Subcutaneous tissue oxygen tension
VEGF	Vascular endothelial growth factor

Hypoxia, a characteristic feature of wounds, stimulates many types of cells, including fibroblasts and macrophages, to produce VEGF. It would be easy to assume, therefore, that wound angiogenesis is a direct consequence of hypoxia. However, clinical experience indicates that tissue ischemia and arterial hypoxemia retard healing while hyperoxia stimulates it.[8–11]

In the current experiments, we used a mouse wound model to examine this paradox. We hypothesized that hyperoxia, which has been shown to stimulate VEGF production,[12] accelerates angiogenesis in wounds, while hypoxia, although it is known to stimulate VEGF production, reduces angiogenesis.

Collagen synthesis and deposition share this paradox with angiogenesis, i.e., hypoxia stimulates the assembly of collagen synthetic mechanisms including increasing collagen mRNA prevalence, procollagen synthesis, and prolyl hydroxylase activity.[13–16] At the same time, however, oxygen is specifically required at rela-

tively high concentrations for posttranslational hydroxylation of proline and lysine, and thus for exportation and cross-linking of collagen. Prolyl hydroxylase, a required enzyme, is an oxygenase, i.e., it requires oxygen as its substrate, and its K_m is relatively high (25 mmHg).[17–19] The net result is that hypoxia increases procollagen synthesis, but imposes a substrate limitation on collagen deposition. A similar scenario appears to occur in angiogenesis in which hypoxia stimulates production of VEGF from macrophages while oxygen is required to complete the process.[6–9,20,21] This similarity seems particularly pertinent because collagen formation and angiogenesis are codependent. As we know from scurvy, angiogenesis is fragile in the absence of active collagen synthesis. New collagen fibers must surround budding vessels in order to prevent them from rupturing when blood pressure is imposed by arterial inflow.[22]

MATERIALS AND METHODS

The University of California, San Francisco Committee on Animal Research, approved all animal procedures and studies.

Murine Matrigel® assay

The Murine Matrigel® angiogenesis assay is technically feasible in mice and pO_2 can be measured within the wound of insertion.[23] Matrigel® (Collaborative Biomedical, Bedford, MA) is a basement membrane complex containing 56% laminin, 31% type IV collagen, 8% entactin, and 5% heparin sulfate proteoglycan. It is isolated from the Engelbreth–Holm–Swarm (EHS) murine tumor of epithelial origin, which produces abundant extracellular matrix.[24,25] The tumor is washed and extracted with urea; the extract is then dialyzed under physiological conditions producing Matrigel®.[25] Matrigel® also contains small quantities of growth factors including transforming growth factor-β (2.3 ng/mL), epidermal growth factor (0.7 ng/mL), insulin-like growth factor one (16 pg/mL), and platelet-derived growth factor (12 pg/mL). However, it stimulates only minimal angiogenesis when implanted without further angiogenic additives. At 4°C, it is a liquid, but it becomes a gel at 22–35°C. Two 1 mL injections of unsupplemented Matrigel® were placed subcutaneously into the dorsum of 5-month-old retired breeder, female Swiss-Webster mice under methoxyflurane inhalation anesthetic (Pitman-Moore, Inc., Mundelein IL). The animals in all arms of the study were fed standard laboratory chow and given water ad libitum.

In some of the experiments (detailed below), recombinant human VEGF-165 (100 ng/plug; R&D Systems, Minneapolis, MN; catalog # 293- VE/CF) was added to each Matrigel® plug prior to subcutaneous injection. Recombinant human VEGF is highly conserved and is angiogenic across species.[6,7] In a separate experiment, polyclonal goat anti-mouse VEGF neutralizing antibody (1 μg/plug; R&D Systems; catalog # AF-493-NA) was added to the Matrigel® prior to subcutaneous injection. As a control for the polyclonal anti-VEGF antibody, the same concentration of protein in the form of normal goat immunoglobulin γ (IgG; 1 μg/plug; R&D Systems; catalog # AB-108-C) was used in an identical fashion. The control for the recombinant human VEGF, anti-VEGF antibody, and IgG groups was phosphate-buffered saline solution-supplemented Matrigel® (100 μg/plug).

Environmental conditions

Experimental groups were exposed to various oxygen concentrations. The groups were as follows: hypoxia: continuous, 13% oxygen (range 12.4–14.8%) at 1.0 atmosphere absolute (ATA; VEGF-supplemented Matrigel® only); control: continuous room air, 21% oxygen at 1.0 ATA; hyperoxia (including four subgroups): 100% oxygen at 1.0 ATA, 2.0 ATA, 2.5 ATA, and 3.0 ATA. Because animals in the 3 ATA group showed significant pulmonary oxygen toxicity, the 3 ATA exposure was abandoned after the first set of experiments (unsupplemented Matrigel®). The animals in the hypoxia and control groups were exposed constantly while the hyperoxic groups were exposed to the treatment conditions for 90 minutes twice daily for 7 days, breathing room air at ambient atmospheric pressure (748–750 mmHg) between treatments.

This treatment regimen was chosen to mimic clinical hyperbaric oxygen treatment (2–2.8 ATA for 90 minutes once or twice daily, depending on facility). Treatments generally cannot exceed 90 minutes twice daily because of oxygen toxicity (seizures, pulmonary edema). A total of 14 treatments was chosen because, clinically, angiogenesis (new granulation tissue) does not become apparent until after about 14 treatments.[26]

Hypoxic animals were housed in a Plexiglas box with a single gas inlet and outlet. Oxygen was delivered at 1.0 L/minute and nitrogen at 4.5 L/minute (18% oxygen) from tanks connected to the chamber by 20 ft of thick-walled polyvinyl tubing. This reliably delivered 13.3 ± 1.3 percent oxygen to the chamber (measured pO_2 was 93.3 ± 0.58 mmHg within the chamber). Carbon dioxide levels were maintained at 0 mmHg within the chamber with a CO_2 absorber (Baralyme, Chemetron Medical Division, St Louis, MO). A Datex Capnomac II (Datex Medical Instruments, Inc, Tewksbury, MA) was used to sample exhaust gas from the oxygen chambers to confirm the exposure to pO_2 and to ensure that CO_2 did not accumulate. To achieve 100% oxygen at ambient atmospheric pressure, animals were housed in a Plexiglas chamber identical to the one used for the hypoxic animals, with oxygen delivered at 5 L/minute during

treatment. A small hyperbaric chamber (Western Hyperbaric Services, San Pablo, CA) was used to deliver 100% oxygen at 2.0, 2.5, and 3.0 ATA.

Tissue oxygen measurement

A polarographic oxygen electrode (Licox pO_2 computer, GMS, Kiel, Germany) was used to measure subcutaneous tissue oxygen tension ($PsqO_2$) in each of the oxygen environments. The probes were calibrated in room air before use and checked for return to baseline calibration after use. The calibration is linear up to a pO_2 of about 400 mmHg. Above that level, calibration was performed to account for deviation from linearity. A 19 g spinal needle was used to place a Luer-hubbed, 15 cm length of oxygen-permeable silicone tubing (Silastic®, Dow Corning, Midland, MI; inner diameter 0.8 mm, outer diameter 1.0 mm) subcutaneously over the dorsum of the mouse. The oxygen electrode and a temperature probe were placed in the tubing, which was flushed with deoxygenated saline to eliminate air bubbles. The system was then allowed to equilibrate. Equilibration criteria were met when the pO_2 remained constant within ± 1 mmHg over 5 minutes. This device measures the average pO_2 within its local tissue environment.[27,28]

Grading of angiogenesis

After 7 days, the gel plugs were removed and samples were fixed in buffered formalin, paraffin embedded, sectioned (4 μm), and stained with hematoxylin and eosin. Sections were graded microscopically at $\times 100$ power to assess the extent of neovascular formation. Grading was stepwise: 0 = no vessels (Figure 1A), 0.5 = scattered vessels (Figure 1B), and 1 = maximal (several) vessels per $\times 100$ field in all quadrants (Figure 1C). With higher magnification of $\times 400$, capillary tube formation could be seen in some sections (Figure 1D). At $\times 1000$ magnifi-

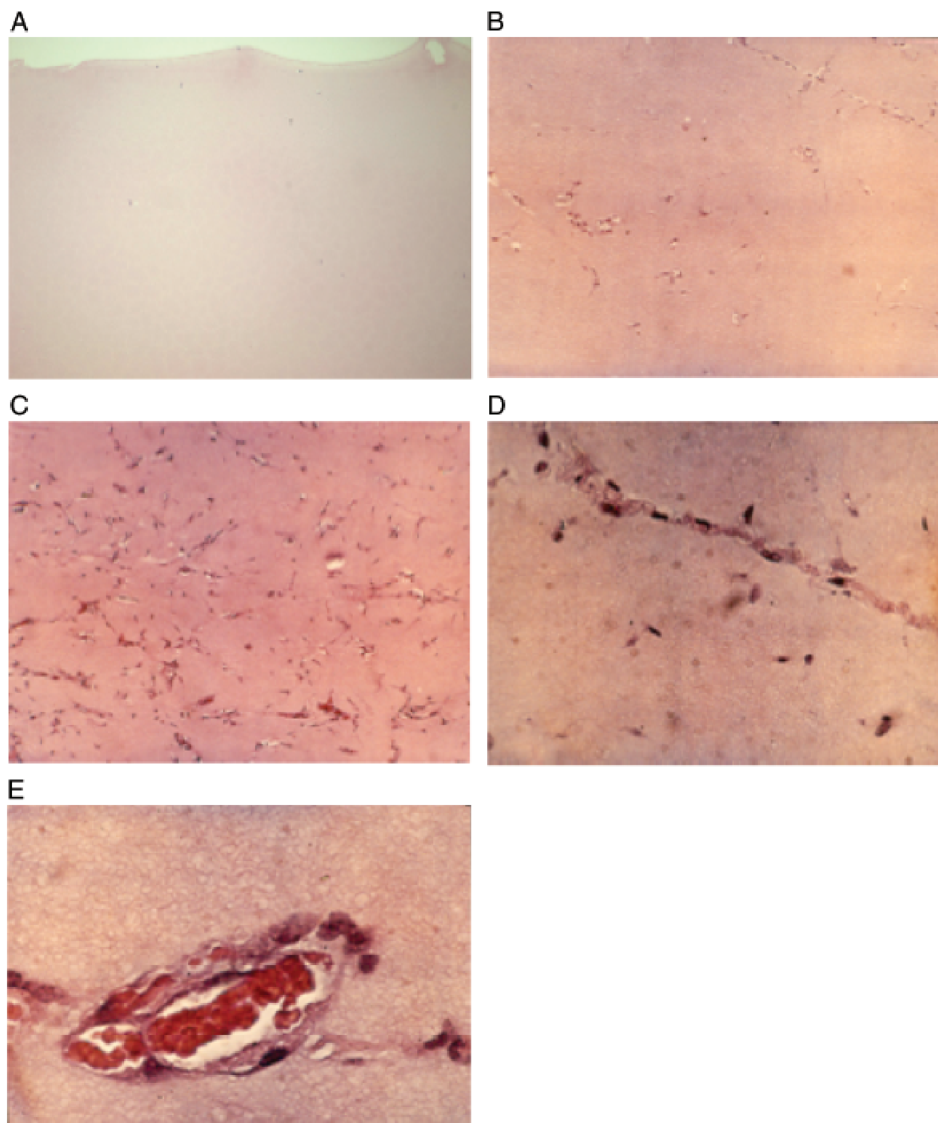

FIGURE 1. Photomicrographs representing the angiogenesis scoring system. (A) Angiogenesis score 0; Matrigel® showing no angiogenesis (Original magnification $\times 100$). (B) Angiogenesis score 0.5; Matrigel® showing scattered neovascularization (Original magnification $\times 100$). (C) Angiogenesis score 1; Matrigel® showing maximal vessels in all quadrants (Original magnification $\times 100$). (D) Matrigel® showing capillary tube formation (Original magnification $\times 400$). (E) Matrigel® showing red blood cells within a capillary lumen (Original magnification $\times 1000$).

cation red blood cells were easily seen within many of the capillaries (Figure 1E). Endothelial cell in-growth into the Matrigel® plugs was also confirmed with factor VIII staining.[23,25] Three independent investigators blinded to the treatment performed histologic assessment. Agreement was near 100 percent. In the few cases where there was a disagreement between raters, the slide was re-reviewed by the three raters and a consensus value recorded. In no case was the difference >1 step.

Statistical analysis

Angiogenesis scores are reported as mean ± SD. Because the data are stepwise (ordinal), the Kruskal–Wallis statistic and the Mann–Whitney test were used to evaluate differences between groups.

RESULTS

Wound $PsqO_2$ values are reported in Figures 2 and 3, along with the angiogenesis scores. Table 1 shows the statistical comparisons for the different angiogenesis scores. Experimental variability was in the range normally seen for in vivo studies of angiogenesis. In the control group (21% oxygen at 1 ATA) without supplemental VEGF, no significant angiogenesis was found (Figure 2). This is consistent with previous studies.[20] Hyperoxia (whether at sea level or at pressure) increased angiogenesis significantly, and in a dose-dependent manner, although there was a ceiling effect.

Addition of VEGF to the Matrigel® stimulated angiogenesis (Figure 3). Continuous hypoxia in this group inhibited angiogenesis significantly as compared with room air-breathing controls. Hyperoxia increased angiogenesis significantly, although the ceiling was quickly reached, and there was no dose dependence for hyperoxia.

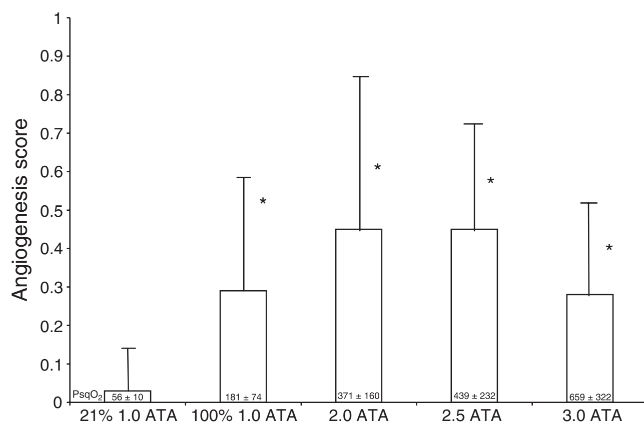

FIGURE 2. Graph of angiogenesis score vs. oxygen treatment group for unsupplemented Matrigel®. Mean $PsqO_2$ is listed for each oxygen group on the data bar. Angiogenesis was significantly increased ($p < 0.05$) in all hyperoxic groups compared with control (normoxia).

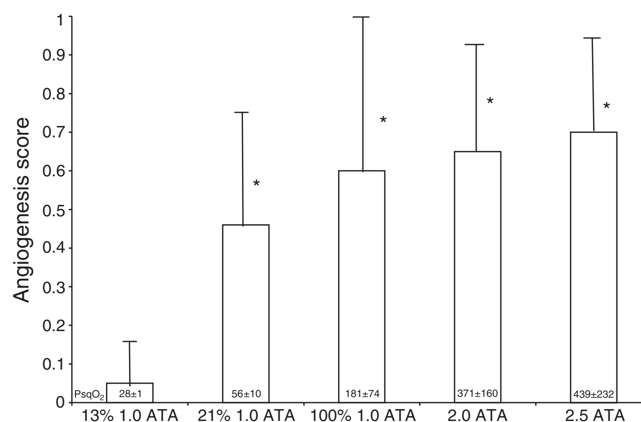

FIGURE 3. Graph of angiogenesis score vs. oxygen treatment group for VEGF-supplemented Matrigel®. Mean $PsqO_2$ is listed for each oxygen group on the data bar. Angiogenesis was significantly increased ($p < 0.05$) in the 2.5 atmosphere absolute (ATA) group compared with normoxic controls; the increase did not reach significance at 1.0 and 2.0 ATA. Angiogenesis was significantly decreased ($p = 0.001$) in the hypoxic group compared with normoxic controls.

VEGF-neutralizing antibody blocked both the effect of VEGF and that of hyperoxia (Table 1). Addition of IgG to the Matrigel® did not stimulate angiogenesis in room air-breathing control animals, while the response to hyperoxia was unaffected (Table 1).

DISCUSSION

These experiments confirm a clinical impression that wound angiogenesis requires oxygen. Our data show that the angiogenic response is dose dependent. Any number of mechanisms might account for the dependency of angiogenesis on oxygen, but one is known. The deposition of the perivascular collagen that is needed to align the new endothelial cells for tube formation cannot occur without oxygen. The pertinent step on which deposition depends is the hydroxylation of collagen proline residues in which the K_m for oxygen is approximately 25 mmHg. Collagen deposition in wounds is seriously impaired by arterial hypoxia and increased by hyperoxia.[29] Clinically, hypoxic wounds are slow to develop new vessels (granulation tissue). We did not evaluate the quality of vessels in the study. However, our impression was that hypoxic animals developed vessels with poor integrity, while hyperoxic animals developed more mature vessels. We plan to examine this formally in a future study.

The remainder of the data seem best explained by known features of oxidant signaling. Reactive oxidant species are required for uncomplicated healing. In particular, immunity to infection requires bactericidal species that are produced in proportion to the ambient pO_2 of leukocytes.[30–32] Hydrogen peroxide enhances colla-

Table 1. In vitro Murine Matrigel® angiogenesis score

	Condition				
	oxygen (%)	ATA	*n*	Mean score ± SD	*p*-value vs. 21%
Matrigel® alone	21	1.0	31	0.03 ± 0.12	
	100	1.0	19	0.29 ± 0.30	0.0002
	100	2.0	21	0.45 ± 0.38	< 0.0001
	100	2.5	10	0.45 ± 0.28	< 0.0001
	100	3.0	9	0.28 ± 0.26	0.0008
Matrigel® + VEGF	13	1.0	10	0.05 ± 0.16	0.001
	21	1.0	12	0.46 ± 0.26	
	100	1.0	10	0.60 ± 0.39	NS (0.31)
	100	2.0	10	0.65 ± 0.24	NS (0.10)
	100	2.5	10	0.70 ± 0.26	0.049
Matrigel® + anti-VEGF ab	21	1.0	17	0.24 ± 0.36	
	100	1.0	20	0.17 ± 0.24	NS (0.80)
	100	2.0	19	0.26 ± 0.35	NS (0.76)
	100	2.5	19	0.21 ± 0.25	NS (0.93)
Matrigel® + IgG	21	1.0	20	0.20 ± 0.30	
	100	1.0	20	0.35 ± 0.33	NS (0.12)
	100	2.0	20	0.45 ± 0.32	0.014
	100	2.5	20	0.47 ± 0.38	0.001

IgG = immunoglobulin G; NS = nonsignificant.

gen mRNA abundance[33] and other cytokines and growth factors depend on oxygen supply.

Hydrogen peroxide at low concentrations stimulates VEGF generation in pertinent cells.[34] Macrophages produce VEGF in proportion to pO_2 and when exposed to low levels of hydrogen peroxide.[35]

The evidence suggests that hypoxia may not be the only stimulus for release of angiogenic signals, including VEGF. Several mechanisms are known, which can stimulate VEGF. One of them is probably pertinent to the present data. Lactate stimulates VEGF release from macrophages. Wounds, of which the Matrigel® plug is an example, characteristically have high lactate levels. The lactate is not the result of hypoxia, because wound cells, including inflammatory and endothelial cells, rely heavily on aerobic glycolysis for their energy production and release lactate regardless of oxygen supply.[32] Thus, hyperoxia does not reduce lactate levels in wounds.[36,37] High lactate also induces collagen synthesis.[38] Although lactate production is not related to hypoxia in this case, it nevertheless shares a similarity with both hypoxia and oxidants. All have a common consequence. They lower the level of oxidized nicotinamide adenine dinucleotide (NAD+). That, in turn, inhibits adenosine diphosphate ribosylation and enhances both collagen deposition and VEGF release.

Our results may also be significant to ischemic but unwounded tissues. Intermittent hyperbaric oxygen increases limb preservation in ischemic diabetic extremities.[39] The implication is that hyperbaric oxygen is angiogenic in that circumstance. The present results support that interpretation.

There is little doubt in clinical practice that oxygen is required for healing and accelerates angiogenesis. Supportive data have been published by Knighton and Hunt, who used rabbit ear chambers and supplemental oxygen at sea-level atmospheric pressure (1 ATA).[5] Bilic and others found proliferation of capillaries when burned rats were exposed to intermittent hyperbaric oxygen.[40] Zhao et al. combined hyperbaric oxygen with a growth factor to accelerate angiogenesis in an ischemic rabbit ear model.[8]

The present study adds another dimension to these previous studies by showing that new vessel growth requires oxygen and that hypoxia inhibits vascular growth even in the presence of added VEGF. Because an oxidant-based mechanism for VEGF release has been reported,[41] it is logically possible that the hyperoxia achieved in these studies might enhance the production of angiogenic stimuli. When anti-VEGF antibody was added to Matrigel®, the oxygen effect was abrogated, although not eliminated, suggesting that there are at least two effects of oxygen. One part of the oxygen effect is mediated through increased VEGF production.[12] Another role of oxygen is to support one or more needs of growing vessels such as hydroxylating proline to convert procollagen to collagen. Future studies will examine these mechanisms.

The inhibition of angiogenesis by hypoxia shown in our study is undoubtedly clinically pertinent. The degree of wound hypoxia associated with a lack of angiogenesis in this model is also commonly found in both chronic and acute wounds in humans and is associated with serious impairment of granulation tissue development.[29,42]

The cyclic nature of the exposure to oxygen used in these experiments may be significant. It may have allowed VEGF, VEGF receptors on endothelial cells, and procollagen to accumulate during the hypoxic phase,

i.e., between hyperoxic exposures.[43,44] Increased oxygen concentration may then have subsequently satisfied other requirements for angiogenesis such as hydroxylation and export of collagen.

In summary, oxygen is essential for angiogenesis in wounds and VEGF plays a key role. Continuous hypoxia inhibits neovascularization even when it is stimulated by exogenous VEGF. Periodic hyperoxia stimulates angiogenesis presumably by satisfying metabolic needs and by enhancing VEGF production. The clinical lesson is clear. Maintenance of adequate tissue pO_2 is critical to wound healing.

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