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### Stem cell mobilization by hyperbaric oxygen

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**Thom, Stephen R., Veena M. Bhopale, Omaid C. Velazquez, Lee J. Goldstein, Lynne H. Thom, and Donald G. Buerk.** Stem cell mobilization by hyperbaric oxygen. *Am J Physiol Heart Circ Physiol* 290: H1378–H1386, 2006. First published November 18, 2005; doi:10.1152/ajpheart.00888.2005.—We hypothesized that exposure to hyperbaric oxygen (HBO<sub>2</sub>) would mobilize stem/progenitor cells from the bone marrow by a nitric oxide (·NO)-dependent mechanism. The population of CD34<sup>+</sup> cells in the peripheral circulation of humans doubled in response to a single exposure to 2.0 atmospheres absolute (ATA) O<sub>2</sub> for 2 h. Over a course of 20 treatments, circulating CD34<sup>+</sup> cells increased eightfold, although the overall circulating white cell count was not significantly increased. The number of colony-forming cells (CFCs) increased from 16 ± 2 to 26 ± 3 CFCs/100,000 monocytes plated. Elevations in CFCs were entirely due to the CD34<sup>+</sup> subpopulation, but increased cell growth only occurred in samples obtained immediately posttreatment. A high proportion of progeny cells express receptors for vascular endothelial growth factor-2 and for stromal-derived growth factor. In mice, HBO<sub>2</sub> increased circulating stem cell factor by 50%, increased the number of circulating cells expressing stem cell antigen-1 and CD34 by 3.4-fold, and doubled the number of CFCs. Bone marrow ·NO concentration increased by 1,008 ± 255 nM in association with HBO<sub>2</sub>. Stem cell mobilization did not occur in knockout mice lacking genes for endothelial ·NO synthase. Moreover, pretreatment of wild-type mice with a ·NO synthase inhibitor prevented the HBO<sub>2</sub>-induced elevation in stem cell factor and circulating stem cells. We conclude that HBO<sub>2</sub> mobilizes stem/progenitor cells by stimulating ·NO synthesis.

nitric oxide; CD34; CD133; CXCR4; cKit; colony-forming cells; progenitor cells

THE GOAL of this investigation was to determine whether exposure to hyperbaric oxygen (HBO<sub>2</sub>) would mobilize bone marrow-derived stem/progenitor cells (SPCs) in humans and animals. Pluripotent SPCs from adults exhibit properties similar to embryonic SPCs and hold promise for treatment of degenerative and inherited disorders (9, 20). Postnatal neovascularization occurs by sprouting of endothelium from preexisting blood vessels (angiogenesis) and by endothelial SPCs released from the bone marrow that home to foci of ischemia in a process termed vasculogenesis (21). SPC mobilization from the bone marrow can be stimulated by peripheral ischemia, vigorous exercise, chemotherapeutic agents, and hematopoietic growth factors (2, 16, 22, 23, 27, 30, 36). SPCs also can be obtained by direct bone marrow harvesting and ex vivo manipulations (10,

28, 32). Hematopoietic SPCs are typically obtained for the purpose of bone marrow transplantation by administration of chemotherapeutic agents and growth factors (36). Utilizing these agents to obtain autologous SPCs for treating disorders such as organ and limb ischemia, and refractory wounds, has been considered, but application is thwarted because of risks such as acute arterial thrombosis, angina, sepsis, and death (7, 20, 21, 27, 29, 30, 36).

Nitric oxide (·NO) plays a key role in triggering SPC mobilization from the bone marrow via release of the stem cell active cytokine, cKit ligand (stem cell factor, SCF) (1, 8). Because HBO<sub>2</sub> can activate ·NO synthase in different tissues, we hypothesized that exposure to HBO<sub>2</sub> may stimulate SPC mobilization to the peripheral circulation (33, 34). In a murine model, we found HBO<sub>2</sub> augments SPC mobilization and recruitment to ischemic wounds and hastens ischemic wound healing (Goldstein LJ, Gallagher K, Baireddy V, Bauer SM, Bauer RJ, Buerk DG, Thom SR, Velazquez OC, unpublished observations). SPCs have been shown to home to ischemic wounds, where they are required for angiogenesis (3).

HBO<sub>2</sub> therapy is administered for a variety of maladies in a hyperbaric chamber where patients breathe pure O<sub>2</sub> at partial pressures up to 3.0 atmospheres absolute (ATA). HBO<sub>2</sub> is used in a standard fashion as prophylactic treatment to reduce the incidence of osteoradionecrosis (ORN) in patients who must undergo surgery involving tissues previously exposed to radiotherapy (6, 15). We obtained peripheral blood samples from normal human volunteers and from a group of patients undergoing prophylactic HBO<sub>2</sub> in anticipation of surgery to reduce their risk for ORN and examined the blood for the presence of SPCs. We then investigated the mechanism for SPC mobilization in mice. Here, we demonstrate that HBO<sub>2</sub> causes rapid SPC mobilization in both humans and mice and that this occurs via a ·NO-dependent mechanism.

#### METHODS

*Stem cell release in humans exposed to HBO<sub>2</sub>.* This protocol was approved by the Institutional Review Board and by the Clinical Trials Scientific Monitoring Committee of the Abramson Cancer Center. Patients are referred to the University of Pennsylvania Institute for Environmental Medicine for prophylactic HBO<sub>2</sub> treatment because of a risk for ORN. A group of these patients was approached, and after informed consent, blood was obtained before and after their first, 10th, and 20th HBO<sub>2</sub> treatment (2.0 ATA O<sub>2</sub> for 2 h). All of these patients

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had undergone radiotherapy for head or neck tumors; none had open ulcerations, nor were they taking corticosteroids or chemotherapeutic agents. On the basis of current standard of care, they received HBO<sub>2</sub> therapy before undergoing oral surgery due to radiation-induced xerostomia and caries. Men ( $n = 18$ ) had an average age of  $56 \pm 2$  (SE) yr, and women ( $n = 8$ )  $53 \pm 4$  yr. Three inside-chamber paramedic attendants, men with an average age of  $48 \pm 3$  yr, also had blood drawn before and after pressurization to 2.0 ATA for 2 h. These individuals served as a control for the effect of pressure vs. hyperoxia, as they breathe air and not pure oxygen inside the hyperbaric chamber. Three normal, healthy human volunteers, two men and one woman with an average age of  $53 \pm 3$  yr, also underwent 2-h exposures to hyperoxia.

Citrate anticoagulated blood (16 ml) was centrifuged through Histopaque 1077 (Sigma) at 400 g for 30 min to isolate leukocytes, and cells were washed in PBS. Where indicated, isolated leukocytes underwent further purification to obtain CD34<sup>+</sup> and CD34<sup>-</sup> cells by using paramagnetic polystyrene beads coated with antibody to CD34 (DynaL Biotech, Lake Success, NY). Isolation was carried out exactly as recommended by the manufacturer except that while cells were attached to the beads they were washed only twice, not three times. Normally, the bead selection system achieves 90% purity for CD34-expressing cells but recovers only ~50% of all CD34<sup>+</sup> cells from a cell suspension. Our goal was to assess the growth potential of the CD34<sup>+</sup> and CD34<sup>-</sup> cells separately. With our modified separation method, the aspirated cells that did not attach to the CD34 antibody-coated beads contained only  $1.4 \pm 0.4\%$  (SE,  $n = 9$ ) of the CD34-expressing cells in the total monocyte population, and the recovered cells detached from the beads were  $75 \pm 4\%$  pure. That is, ~25% of the monocytes used in the "CD34<sup>+</sup>" cultures did not express CD34.

For flow cytometry analysis, washed monocytes were suspended in 250  $\mu$ l PBS + 0.5% BSA. Cells were first incubated with rabbit IgG (250  $\mu$ g/ml) for 5 min at 4°C to block Fc receptors and then incubated with a saturating concentration of R-phycoerythrin (RPE)-conjugated mouse anti-human CD34 (Clone 581, a class III CD34 epitope; BD Pharmingen, San Jose, CA), fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CXCR4 (R and D Systems, Minneapolis, MN), and either allophycocyanin (APC)-conjugated mouse anti-human vascular endothelial growth factor-2 receptor (VEGFR-2) (R and D Systems) or APC-conjugated CD133 (Miltenyi Biotec, Auburn, CA) for 30 min at 4°C. Isotype-matched mouse immunoglobulin served as control. Cells were then washed with PBS, and residual erythrocytes were lysed by incubation in 155 mM ammonium chloride, 0.1 mM EDTA and 10 mM sodium carbonate (pH 7.2), centrifuged, and resuspended in PBS. Flow cytometry was performed using a FACScan flow cytometer (Becton Dickinson) at the Abramson Cancer Center Flow Cytometry Core facility. Monocytes were gated on the basis of forward and side laser light scattering, and 100,000 gated cells were analyzed for expression of cell surface markers that may be present on SPCs.

For the colony-forming cell (CFC) assays, monocytes were washed and then suspended in Metho-Cult colony assay medium (StemCell Technologies, Vancouver, BC, Canada), which contains methylcellulose, L-glutamine, fetal bovine serum, bovine serum albumin, recombinant human stem cell factor, granulocyte-monocyte colony stimulating factor, interleukin-3 (IL-3), and erythropoietin. Cultures were initiated with 1 ml of suspension/well of a six-well Petri plate and incubated at 37°C, air with 5% CO<sub>2</sub>, in a fully humidified atmosphere. Nonselected monocytes were cultured at a concentration of 100,000 cells/plate, and isolated CD34<sup>+</sup> cells were cultured at 50,000 cells/plate. Colonies were apparent and counted using an inverted stage microscope at 14 days.

The phenotype of progeny cells from CFCs plates were analyzed by flow cytometry and confocal microscopy. Cells on CFC plates were harvested by first mixing 5 ml PBS + 0.5 mM EDTA with the semi-soft Metho-Cult medium in plates and then centrifuging at 500 g for 5 min. The cell pellet was washed once in PBS + 0.5% BSA,

and one aliquot of cells was characterized by flow cytometry as described above. A second aliquot of cells was resuspended in growth medium and cultured in 24-well plates. Cells were suspended in 60% Dulbecco's modified Eagle's medium (low glucose; GIBCO BRL, Rockford, MD), 40% MCB2-201 medium (Sigma, St. Louis, MO), and the following supplements (all purchased from Sigma): 1 $\times$  insulin-transferrin-selenium, linolenic acid-BSA, 10<sup>-9</sup> M dexamethasone, 10<sup>-4</sup> M ascorbic acid-2-phosphate, 100 U penicillin, and 1,000 U streptomycin. After growth to confluence, cells were scraped from plates, washed in PBS, and spotted onto polylysine-coated microscope slides. Cells were fixed with 1% paraformaldehyde for 10 min and blocked for 1 h at 4°C with Tris-buffered saline (pH 8.3) containing 10 mM Tris, 250 mM NaCl, 0.3% Tween 20, and 1% BSA. Cells were then covered with 50  $\mu$ l 1:1,000 dilution of mouse anti-human von Willebrand factor (BD Pharmingen) made up in PBS + 0.5% BSA for 1 h at 4°C, washed twice with PBS, and then counterstained for 1 h at 4°C with a 1:2,500 dilution of anti-mouse antibody conjugated to Cy3 and FITC-conjugated *Ulex europaeus* agglutinin (Sigma). Cells were examined with a Bio-Rad Radiance 2000 attached to a Nikon TE 300 inverted stage confocal microscope that was operated with a red diode laser at 638 nm and krypton lasers at 488 and 543 nm.

**Mouse studies.** Wild-type and endothelial  $\cdot$ NO synthase knockout (eNOS KO) mice (*Mus musculus*) were purchased (Jackson Laboratories, Bar Harbor, ME), fed a standard rodent diet and water ad libitum, and housed in the animal facilities of the University of Pennsylvania. Mice were exposed to HBO<sub>2</sub> for 90 min following our published protocol (27, 28). In select studies, wild-type mice were pretreated with intraperitoneal N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME), 40 mg/kg, at 2 h before pressurization. Blood was obtained from anesthetized mice [intraperitoneal administration of ketamine (100 mg/kg) and xylazine (10 mg/kg)] by aortic puncture, and bone marrow was harvested by clipping the ends off a femur and flushing the marrow cavity with 1 ml PBS. Leukocytes were isolated in a procedure essentially the same as that described above for human cells, except that blood cells were centrifuged through Histopaque 1083 (Sigma). Antibody staining of cell surface markers was performed as described above by using FITC-conjugated rat anti-mouse stem cell antigen-1 (Sca-1) and RPE-conjugated rat anti-mouse CD34 (both from BD Pharmingen). Mouse stem cell factor was measured using the Quantikine M immunoassay kit from R and D Systems following the manufacturer's instructions.

Bone marrow  $\cdot$ NO level was measured by placing microelectrodes selective for  $\cdot$ NO into the distal femur marrow cavity. Mice were anesthetized, the femurs were exposed, and a 25-gauge needle was used to bore a hole through cortical bone. Nafion-coated  $\cdot$ NO microelectrodes, fabricated from flint glass micropipettes as described in a prior publication (33), were placed within the cavity and held in place by a micromanipulator arm assembly. The mice were then placed within the hyperbaric chamber for exposure to HBO<sub>2</sub>. In selected studies, while breathing just air and not HBO<sub>2</sub>, mice received an intraperitoneal dose of sodium nitroprusside (4–8 mg/kg) to assess whether this manipulation would alter bone marrow  $\cdot$ NO concentration and mobilize SPCs.

**Statistics.** Statistical analysis of human stem cell numbers was carried out by repeated-measures ANOVA followed by the Dunnett test (SigmaSTAT, Jandel Scientific). CFCs before and after hyperoxia were analyzed by *t*-test, and mouse stem cell mobilization were analyzed by ANOVA followed by Dunn's test. The level of significance was taken as  $P < 0.05$ , and results are expressed as means  $\pm$  SE.

## RESULTS

**SPCs mobilization in humans.** Blood from patients was obtained before and after the first, 10th, and 20th hyperbaric treatments for ORN prophylaxis (the standard preoperative course of therapy is 20 treatments). Blood leukocytes were

harvested and analyzed for the presence of SPCs on the basis of flow cytometry and CFCs.

Results from flow cytometry indicated that there was a range of responses to HBO<sub>2</sub>, and to exhibit this, results from three different patients are shown in Figs. 1–3. Figure 1A shows a typical scatterdot plot from one cell sample. Before patients were exposed to HBO<sub>2</sub>, very few blood cells were positive for CD34, the most commonly used cell surface marker for SPCs (20). There were also few cells that expressed VEGFR-2, the receptor for stromal-derived growth factor (CXCR4), or another SPC surface marker, CD133 (20). These markers were also rarely present on cells from the paramedic attendants inside the hyperbaric chamber, who served as controls for the effect of pressure per se in this study (e.g., Fig. 1C; CD133 data not shown). A comparison of Fig. 1, D and G, shows that the number of cells expressing CD34 was increased in blood after the first HBO<sub>2</sub> treatment.

Subsequent to each HBO<sub>2</sub> treatment, we found a small elevation in a population of cells with moderately elevated CD34 expression (exhibiting intensity at between 10 and ~50) and another population with higher intensity of ~100 to 1,000. The dot plots in Fig. 1, E vs. H, show the pattern of CD34 and VEGFR-2 expression for gated cells. Figure 1H shows a population of cells expressing both surface markers (top right quadrant), and histograms (Fig. 1, F and I) show the expression of VEGFR-2 on cells before and after the patient's first HBO<sub>2</sub> treatment. In all 26 patients, we found the majority of high-intensity CD34<sup>+</sup> cells also expressed VEGFR-2 at an intensity between 10 and 100.

Figure 2 exhibits responses in a patient before and after his 10th HBO<sub>2</sub> treatment. Cell expression of CD34 was elevated before the 10th treatment, and this will be discussed further below (see Fig. 4). The CD34<sup>+</sup> population in this patient exhibited somewhat lower surface expression (intensity ~100)

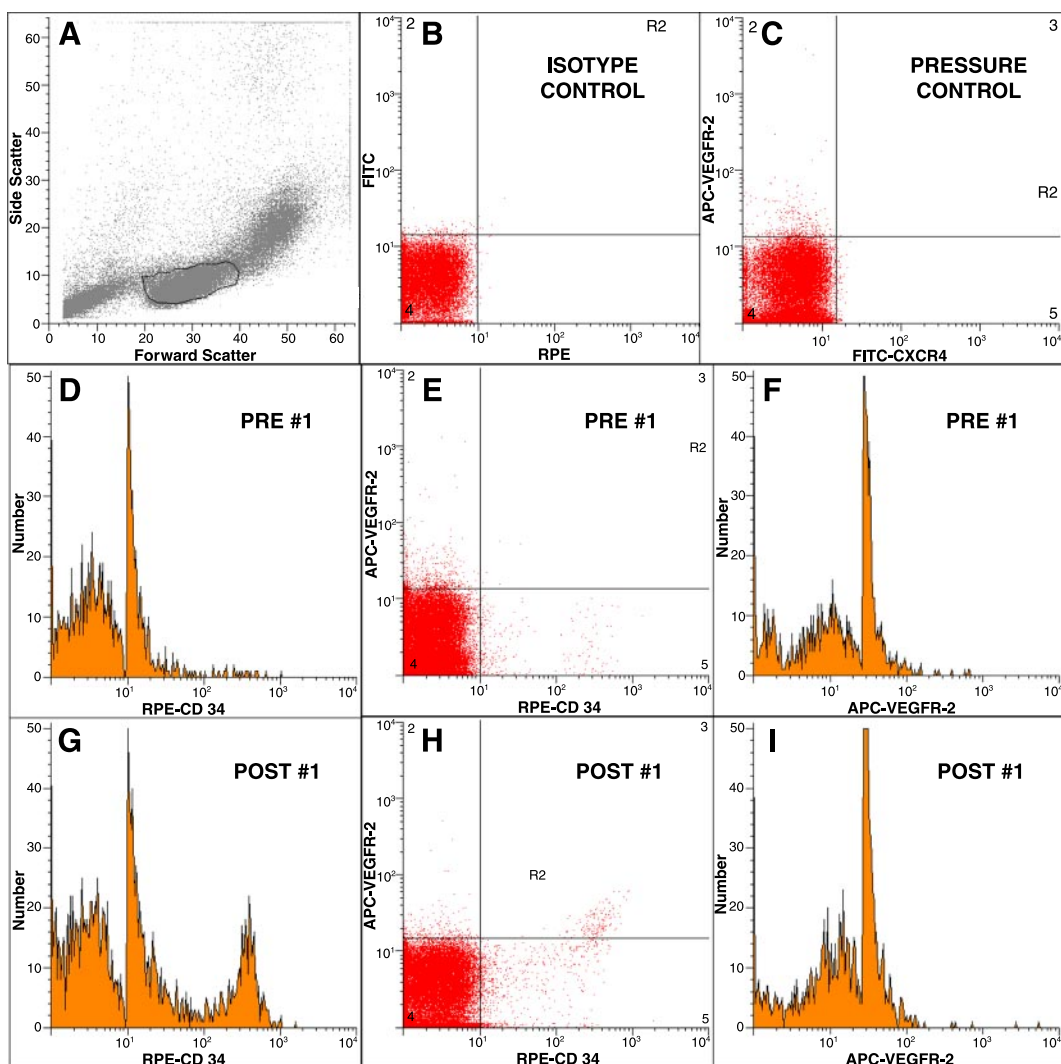


Fig. 1. Flow cytometry analysis of human leukocytes. Data from a pressure-control subject (paramedic) and a patient before and after their first hyperbaric oxygen (HBO<sub>2</sub>) treatment. A: typical forward- and sidescatter dot plot; the black circle indicates the gated cell population analyzed for cell surface markers. B: gated sampling of cells incubated with isotype-matched control mouse antibodies conjugated to fluorescein isothiocyanate (FITC) or R-phycoerythrin (RPE). C: dot plot for cells from a paramedic, pressure control individual stained for vascular endothelial growth factor-2 receptor (VEGFR-2) and the receptor for stromal-derived growth factor (CXCR4). APC, allophycocyanin. D–I: data obtained from 100,000 gated cells from a patient stained for both CD34 and VEGFR-2. The second row of plots (D–F) exhibits expression patterns for cells pre-HBO<sub>2</sub>, and the third row of plots (G–I) shows the gated cell population post-HBO<sub>2</sub> treatment.

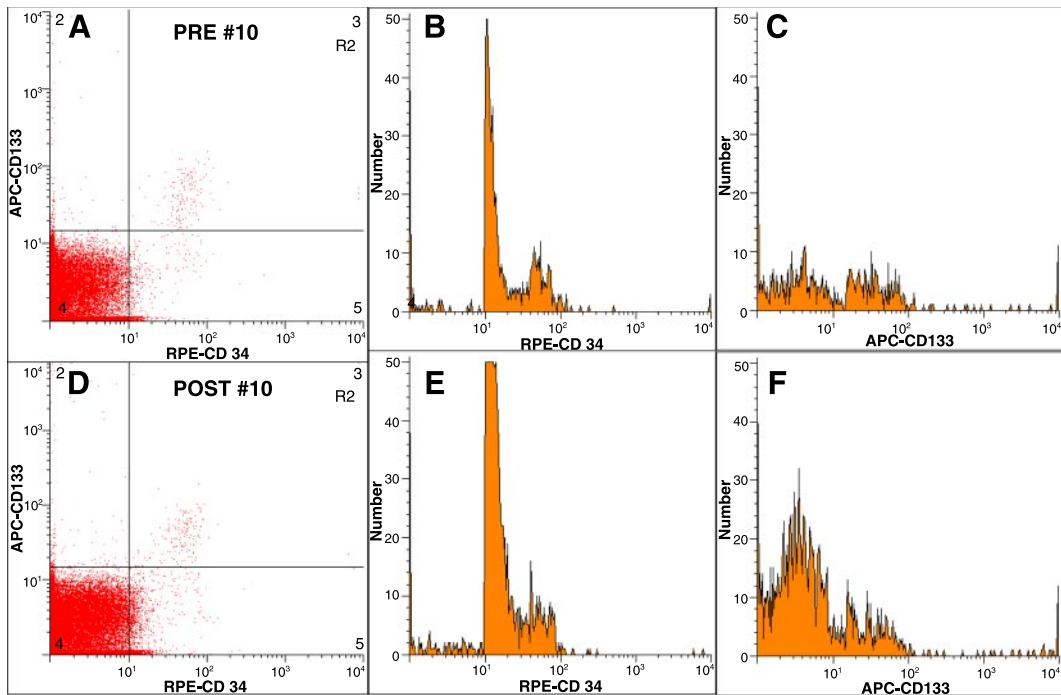


Fig. 2. Flow cytometry analysis of 100,000 human leukocytes gated as shown in Fig. 1 that were stained for CD34 and CD133. Data are from 1 patient before the 10th HBO<sub>2</sub> treatment (A–C) and after the 10th HBO<sub>2</sub> treatment (D–F).

than the patients shown in Figs. 1 and 3, something we observed in a total of three patients. Circulating endothelial cells express CD34, and they may express VEGFR-2; thus, to more carefully discern whether HBO<sub>2</sub> mobilized SPCs, we also probed cells for expression of CD133 and CXCR4. CD133 is not expressed by endothelial cells, and CXCR4 is expressed on

a subset of SPCs (5, 13, 17, 20). A population of cells expressing both CD34 and CD133 can be seen in Fig. 2, A and D (top right quadrant before and after the 10th treatment). Histograms for CD34 and CD133 expression on circulating cells are also shown in Fig. 2. Figure 3 shows responses in a

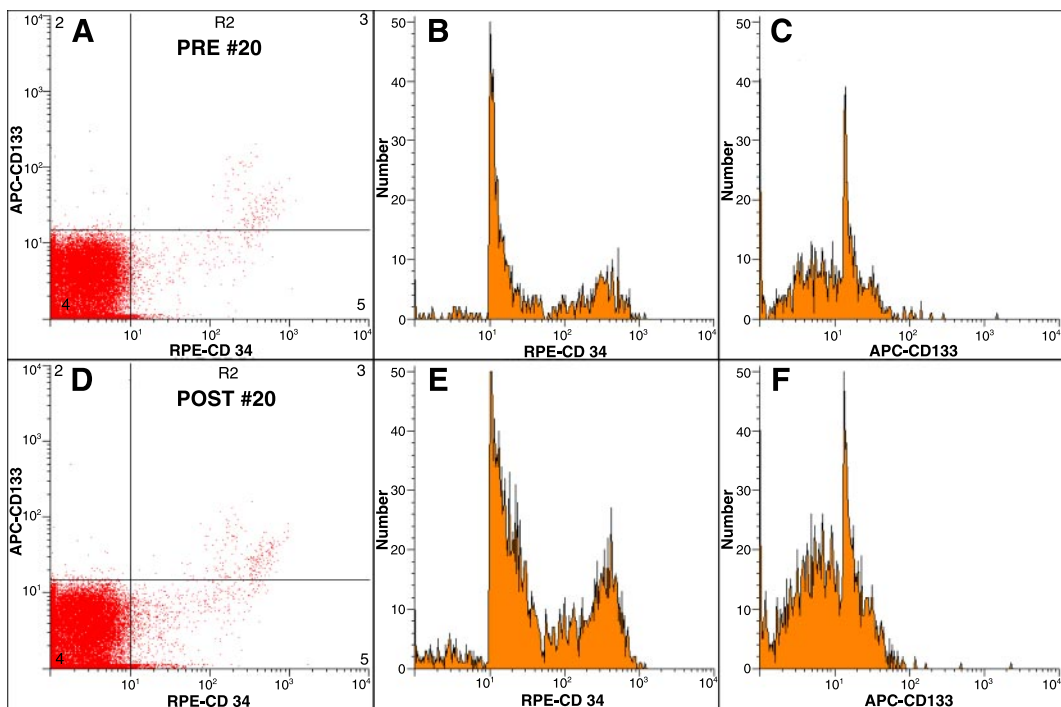


Fig. 3. Flow cytometry analysis of 100,000 human leukocytes gated as shown in Fig. 1 that were stained for CD34 and CD133. Data are from 1 patient before the 20th HBO<sub>2</sub> treatment (A–C) and after the 20th HBO<sub>2</sub> treatment (D–F).

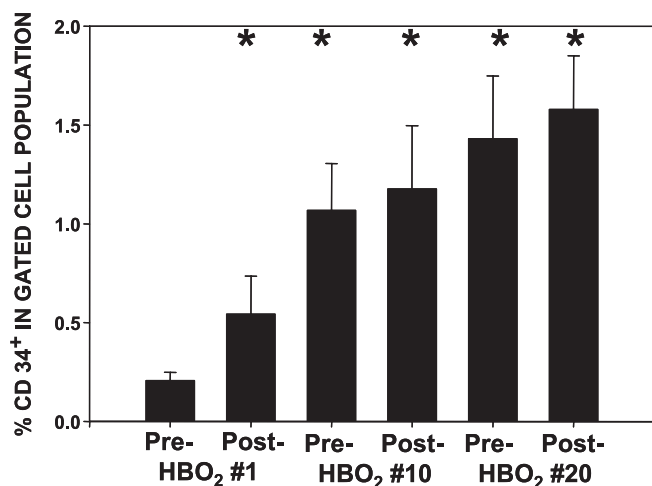


Fig. 4. Mean CD34<sup>+</sup> population in blood of humans before and after HBO<sub>2</sub> treatments. Data are the fraction of CD34<sup>+</sup> cells within the gated population using leukocytes obtained from 26 patients before and after their 1st, 10th, and 20th HBO<sub>2</sub> treatment. \*Repeated-measures one-way ANOVA,  $P < 0.05$  vs. the pre-HBO<sub>2</sub> first treatment value.

We defined CD34<sup>+</sup> cells as having fluorescence intensity above 10. As shown in Fig. 4, there were persistent elevations in the circulating CD34<sup>+</sup> populations subsequent to the first HBO<sub>2</sub> treatment. However, the number of leukocytes in peripheral blood was not significantly different pre- vs. post-HBO<sub>2</sub> ( $6.8 \pm 0.3 \times 10^3/\mu\text{l}$ , 27% mononuclear preexposure; and  $6.7 \pm 0.8 \times 10^3/\mu\text{l}$ , 28% mononuclear, postexposure), consistent with our previous observations (35). The fraction of CD34<sup>+</sup> cells in the gated population was  $0.20 \pm 0.05\%$  (SE) before the first HBO<sub>2</sub> treatment and  $1.58 \pm 0.27\%$  after the 20th HBO<sub>2</sub> treatment, an eightfold elevation. SPC mobilization was due to exposure to hyperoxia, and not just pressure, because no augmentation of circulating CD34<sup>+</sup> cells was observed in three paramedic medical attendants who assisted patients inside the hyperbaric chamber (who breathe air, not pure oxygen, while at 2.0 ATA). Figure 1C shows a cell sample obtained after one paramedic underwent pressurization, and the CD34<sup>+</sup> population looked similar to that shown in Fig. 1, D and E.

The number of CD34-expressing cells increased significantly between the 1st and 10th treatment. There was a trend toward a further increase (not significant) between the 10th and 20th treatment. Although the numbers of CD34<sup>+</sup> cells were not significantly different before vs. after the 10th and 20th treatments, by the 20th treatment the subset of CD34<sup>+</sup> cells that also expressed CXCR4 was significantly higher compared with the dually positive population before HBO<sub>2</sub> started. These results are shown in Fig. 5.

Circulating SPCs were also measured in three healthy human subjects before and after a single 2-h exposure to either 1 or 2 ATA O<sub>2</sub>. There was no significant alteration in circulating SPCs due to exposure to 1 ATA O<sub>2</sub> (data not shown), but we found a threefold increase due to exposure to 2 ATA O<sub>2</sub>, a significantly more robust response to a single HBO<sub>2</sub> exposure than observed in the patient population described in Fig. 4. Before exposure to 2.0 ATA O<sub>2</sub>, the mean fraction of CD34<sup>+</sup> cells was  $0.20 \pm 0.02\%$ , and subsequent to hyperoxia at 2 ATA O<sub>2</sub>, the mean fraction of CD34<sup>+</sup> cells was  $0.67 \pm 0.03\%$  ( $P < 0.05$ ).

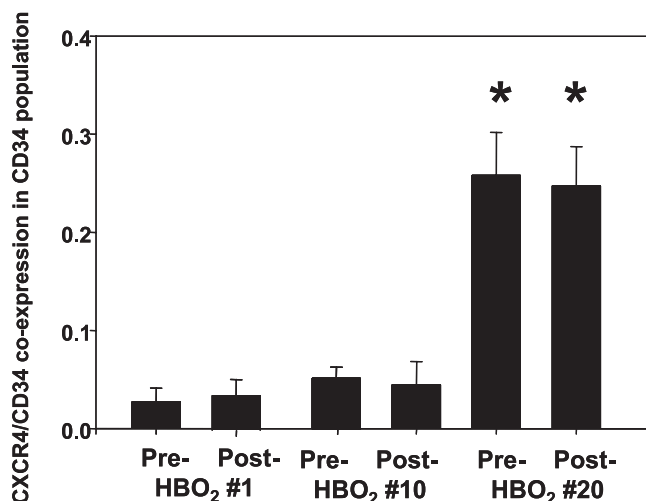


Fig. 5. Proportion of circulating CD34-expressing cells that also express CXCR4 before and after the 1st, 10th, and 20th HBO<sub>2</sub> treatments ( $n = 26$  patients). \*Repeated-measures one-way ANOVA,  $P < 0.05$  vs. the pre-HBO<sub>2</sub> first treatment value.

An alternative approach to assess SPCs was to determine the number of CFCs in peripheral blood. As shown in Fig. 6, CFCs were significantly increased in response to each exposure to HBO<sub>2</sub>. Of note, we did not find elevations in CFCs before the 10th and 20th treatments, although the numbers of CD34<sup>+</sup> cells were elevated (Fig. 4). As these trials were conducted in unselected monocytes, a series of trials was conducted after CD34-expressing monocytes were separated using paramagnetic beads coated with antibody to CD34 (see METHODS). This procedure was carried out on cells from nine patients before and after their 20th HBO<sub>2</sub> treatment. We anticipated better growth in the enriched population, so cells were plated at a reduced density, 50,000 per plate, vs. the 100,000 per plate as in Fig. 6. Before treatment, there were  $12 \pm 1$  colonies/plate, and after HBO<sub>2</sub>,  $23 \pm 2$  colonies grew ( $P < 0.05$ ), whereas in the CD34-negative fraction,  $11 \pm 1$  colonies/plate grew before

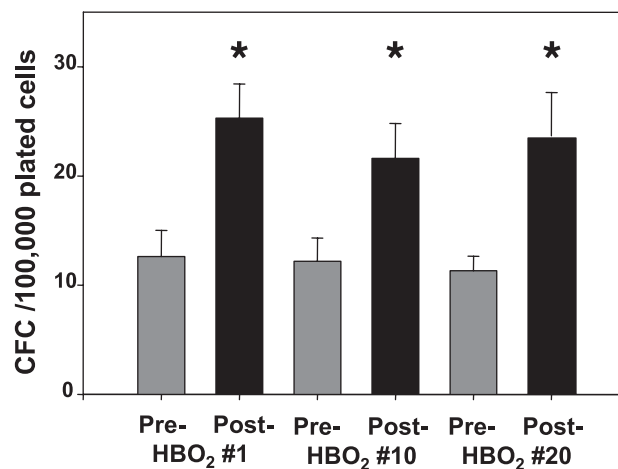


Fig. 6. Colony-forming cells (CFCs) in blood of humans before and after HBO<sub>2</sub> treatments. Data are the colonies counted after a 14-day incubation (all colonies had a myeloid appearance). \*Significant difference by *t*-test performed on each data set pre/post-1st treatment,  $P = 0.036$ ; pre/post-10th treatment,  $P = 0.041$ ; pre/post-20th treatment,  $P = 0.049$ .

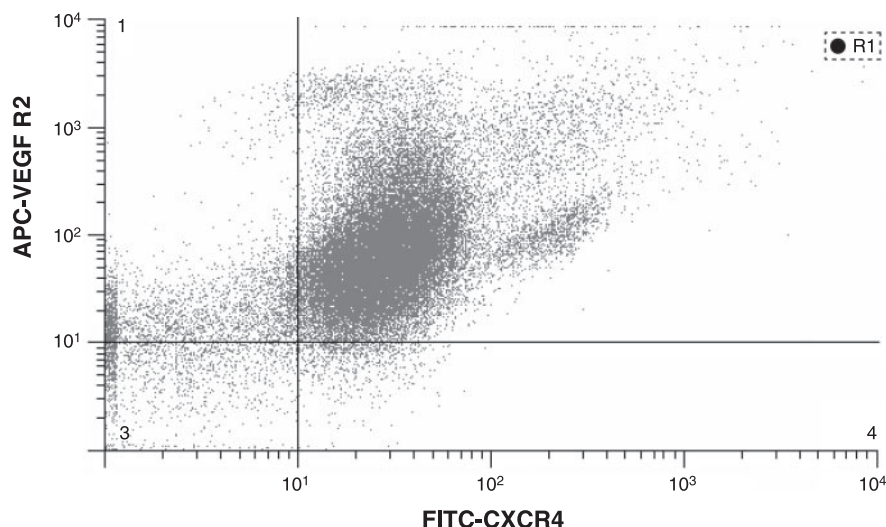


Fig. 7. Flow cytometry scatterplot of progeny cells obtained from CFC plates from 1 patient after the 10th HBO<sub>2</sub> treatment.

treatment and  $11 \pm 1$  colonies/plate (no significant difference) grew after HBO<sub>2</sub>.

The phenotype of progeny cells from a total of 14 patients was analyzed by flow cytometry. Cells were harvested, and expression of CXCR4 and VEGFR-2 was assessed. Figure 7 shows a typical expression pattern, and we could identify no discernible difference whether cells were cultured after the 1st, 10th, or 20th HBO<sub>2</sub> treatment. Progeny cells were also subcultured and examined by confocal microscopy. Approximately 10% of cells heavily expressed von Willebrand factor and stained positive for *Ulex europaeus* lectin, suggesting that a subset of the mobilized SPCs are endothelial progenitors.

**Studies in mice.** SPCs in peripheral blood of mice were assessed as cells that coexpressed CD34 and Sca-1. In preliminary trials, we found that the most effective pressure for increasing circulating SPCs in mice was 2.8 ATA O<sub>2</sub>. Exposure to 100% O<sub>2</sub> at ambient pressure and exposure to a pressure control, 2.8 ATA pressure by using a gas containing 7.5% O<sub>2</sub> (so that O<sub>2</sub> partial pressure was the same as ambient air, 0.21 ATA O<sub>2</sub>), did not stimulate SPC mobilization (Fig. 8). If leukocytes were harvested immediately after the HBO<sub>2</sub> exposure, there was a significant increase in CD34<sup>+</sup>/Sca-1<sup>+</sup> cells (Fig. 8).

There is precedent for rapid mobilization of stem cells from bone marrow, but most emigration is believed to occur after a period of cell proliferation within the marrow niche (16, 22). We found that the number of circulating SPCs peaked at 16 h after mice were exposed to 2.8 ATA O<sub>2</sub>, and if mice were exposed to 2.8 ATA O<sub>2</sub> for 90 min on 2 successive days, the number increased even more (Fig. 8). There was no additional increase in peripheral blood SPCs if mice were exposed to more than two HBO<sub>2</sub> treatments. The leukocyte count in peripheral blood and bone marrow did not increase in response to HBO<sub>2</sub>, but there was a significant elevation in CFCs in both blood and bone marrow (Table 1). Soluble kit ligand (stem cell factor, SCF) was significantly elevated in peripheral blood of HBO<sub>2</sub>-exposed mice (Table 1).

A series of studies was carried out to evaluate whether SPC mobilization was a ·NO-mediated response. We found that SPCs were not mobilized in a group of eNOS knockout mice. Air-exposed eNOS knockout mice exhibited more Sca-1/CD34 dual-positive cells in the gated cell population than did wild-type mice,  $0.27 \pm 0.05\%$  ( $n = 4$ ), but there was no evidence

of stem cell mobilization in response to HBO<sub>2</sub>. The cell level 16 h after knockout mice were exposed to 2.8 ATA O<sub>2</sub> for 90 min was  $0.26 \pm 0.08\%$  ( $n = 5$ ). We also found that if wild-type mice were injected before HBO<sub>2</sub> with the nonspecific NO

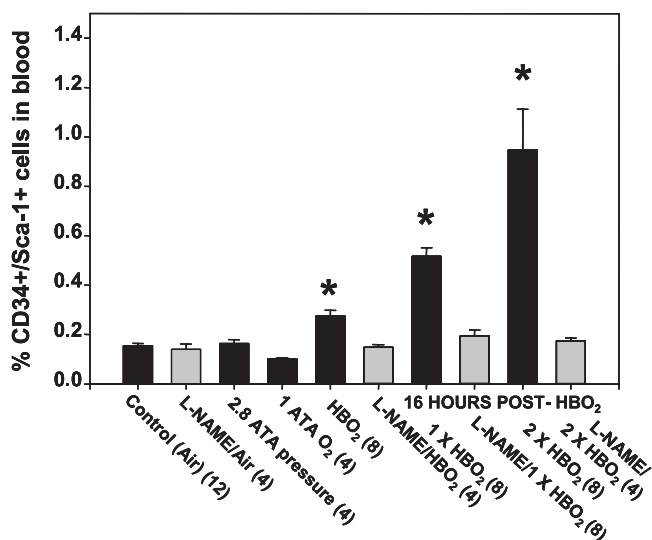


Fig. 8. Mean CD34<sup>+</sup>/Sca-1<sup>+</sup> cells in blood from mice undergoing HBO<sub>2</sub>. *Left to right:* Control (Air), mice were not exposed to pressure or hyperoxia; L-NAME/Air, mice received 40 mg/kg ip N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME) 3.5 h before cells were harvested; 2.8 ATA pressure, mice were exposed to 2.8 atmospheres absolute (ATA) pressure using a gas containing only 7.5% O<sub>2</sub> (0.21 ATA O<sub>2</sub>) for 90 min before cells were harvested; 1 ATA O<sub>2</sub>, mice were exposed to 100% O<sub>2</sub> at ambient pressure for 90 min before cells were harvested; HBO<sub>2</sub>, mice were exposed to 2.8 ATA O<sub>2</sub> for 90 min, and cells were harvested immediately (within 30–90 min) after depressurization; L-NAME/HBO<sub>2</sub>, mice received 40 mg/kg ip L-NAME 2 h before a 90-min exposure to 2.8 ATA O<sub>2</sub>, and then cells were harvested (in 30–90 min); 16 h post-HBO<sub>2</sub> with 1 × HBO<sub>2</sub>, mice were exposed for 90 min to 2.8 ATA O<sub>2</sub> and then left to breathe air for 16 h before cells were harvested; 16 h post-HBO<sub>2</sub> with L-NAME/1 × HBO<sub>2</sub>, mice received 40 mg/kg ip L-NAME 2 h before a 90-min exposure to 2.8 ATA O<sub>2</sub>, and then cells were harvested 16 h after depressurization; 16 h post-HBO<sub>2</sub> with 2 × HBO<sub>2</sub>, mice received two 90-min exposures to 2.8 ATA O<sub>2</sub> separated by 24 h, and cells were harvested 16 h after the 2nd HBO<sub>2</sub> treatment; 16 h post-HBO<sub>2</sub> with L-NAME/2 × HBO<sub>2</sub>, mice received 40 mg/kg L-NAME ip 2 h before each HBO<sub>2</sub> treatment, and cells were harvested 16 h after the 2nd HBO<sub>2</sub> exposure. All data are means ± SE;  $n =$  no. of mice in each sample. \* $P < 0.05$  vs. the control group (1-way ANOVA).

Table 1. Data from mice

	Control	2.8 ATA O <sub>2</sub>
Blood monocytes/ $\mu$ l	2,644 $\pm$ 306	2,103 $\pm$ 297
Marrow leukocytes/tibia	3.2 $\pm$ 2.2 $\times$ 10 <sup>7</sup>	2.9 $\pm$ 2.7 $\times$ 10 <sup>7</sup>
CFCs/50,000 blood leukocytes	2.6 $\pm$ 0.3	4.8 $\pm$ 1.4*
CFCs/50,000 marrow leukocytes	17.0 $\pm$ 1.2	26.2 $\pm$ 1.5*
Plasma SCF, pg/ml	42.6 $\pm$ 2.8	59.5 $\pm$ 0.8*
Plasma SCF (if mice pretreated with L-NAME), pg/ml	39.4 $\pm$ 1.9	42.4 $\pm$ 2.5

Data are means  $\pm$  SE for studies conducted with air-breathing mice (control) and mice killed 16 h after one hyperbaric oxygen (HBO<sub>2</sub>) treatment (6 mice in each group). L-NAME, N<sup>G</sup>-nitro-L-arginine methyl ester; ATA, atmospheres absolute. \*Significant difference by *t*-test: *P* = 0.019 for blood colony-forming cell (CFC) data; *P* = 0.001 for marrow CFC data; *P* = 0.020 for plasma stem cell factor (SCF) data.

synthase inhibitor L-NAME, SPC mobilization did not occur after any of the three different HBO<sub>2</sub>-exposure protocols (Fig. 8). Peripheral blood SCF was not elevated in L-NAME-treated, HBO<sub>2</sub>-exposed mice (Table 1).

Given that  $\cdot$ NO appears to be involved with SPC mobilization, we examined whether administration of a  $\cdot$ NO-donating agent might have an effect similar to HBO<sub>2</sub>. We first examined the alterations in bone marrow  $\cdot$ NO concentrations that resulted from intraperitoneal administration of SNP. We started with a dose of 4 mg/kg, which is anticipated to reduce blood pressure by  $\sim$ 25% for 1 h (31). Figure 9 shows a typical response and also the mean response from three different trials. The  $\cdot$ NO concentration reached a peak of 39  $\pm$  10 nM and returned to baseline by 15 min after SNP injection. Also shown in Fig. 9 is a typical response to HBO<sub>2</sub>, which is consistent with unpublished observations (Goldstein LJ, Gallagher K, Baireddy V, Bauer SM, Bauer RJ, Buerk DG, Thom SR, and Velazquez OC). HBO<sub>2</sub> caused a rapid elevation in bone marrow  $\cdot$ NO that reached a plateau of 1,008  $\pm$  255 nM (*n* = 3), and this level persisted for the duration of the hyperoxic exposure. With the nominal effect of SNP at 4 mg/kg, we carried out four trials using 8 mg/kg, which is approximately three-quarters of the LD<sub>50</sub> for mice (39). Bone marrow  $\cdot$ NO was elevated by 38  $\pm$  12 nM. We also examined circulating SPCs in mice 90 min after SNP administration and found the gated cell Sca-1/CD34

dual-positive population to be 0.11  $\pm$  0.02%, insignificantly different from control.

## DISCUSSION

The results from this study demonstrate that exposure to HBO<sub>2</sub> will cause rapid mobilization of SPCs in humans, and the number of SPCs remain elevated in blood over the course of 20 HBO<sub>2</sub> treatments. On the basis of the responses in normal human controls, it appears that previous exposure to radiation diminishes the response to one HBO<sub>2</sub> treatment. Radiotherapy is known to reduce the mobilization that occurs in response to chemotherapeutic agents and growth factors (19, 25).

Studies in mice indicate that HBO<sub>2</sub> stimulates SPCs mobilization, although the dose response may differ from that observed in humans. We did not systematically examine the time course or dose response for SPC mobilization by HBO<sub>2</sub> in humans. A typical pattern for scientific/medical discovery is to carry out studies in model systems and then verify their occurrence in human beings. In our study, we made our initial observations of SPC mobilization by HBO<sub>2</sub> in humans and then investigated the responses in animals to elucidate the mechanism. In fact, had we started our work with animals, we may not have made our discovery because the HBO<sub>2</sub> protocol typically used (2.0 ATA O<sub>2</sub> for 1–2 h) causes only a rather nominal effect in mice. We found that the most effective pressure for increasing circulating SPCs in mice was 2.8 ATA O<sub>2</sub>.

Observations with eNOS knockout mice and the inhibitory effect of L-NAME in wild-type mice indicate that HBO<sub>2</sub> mobilized SPCs by a  $\cdot$ NO-dependent mechanism. HBO<sub>2</sub> elevates SCF in peripheral blood, and this too is inhibited by L-NAME. These findings are consistent with published work showing that stimulation of bone marrow  $\cdot$ NO synthase will activate metalloproteinase-9 to cleave SCF from its membrane linkage, thus allowing for SCF-mediated SPCs mobilization (1, 8). HBO<sub>2</sub> causes a significant elevation in bone marrow  $\cdot$ NO concentration that cannot be replicated with infusion of SNP.

In patients, there was a significant increase in numbers of CD34<sup>+</sup> cells between the 1st and 10th hyperbaric treatment. In mice, we found that two treatments yielded significantly greater mobilization than one, but no further increase occurred

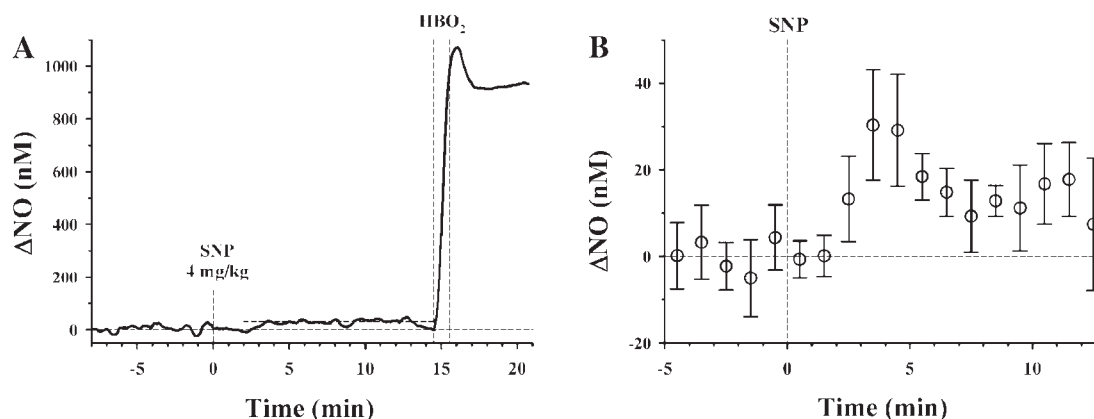


Fig. 9. Nitric oxide ( $\cdot$ NO) concentration in mouse bone marrow. A: an example of the elevation in  $\cdot$ NO after 4 mg/kg ip sodium nitroprusside (SNP). Once the  $\cdot$ NO level returned to baseline, the mouse was pressurized to 2.8 ATA O<sub>2</sub>, and the  $\cdot$ NO level was found to increase markedly. A very similar pattern of responses was observed in 3 separate trials. B: average ( $\pm$ SE) elevation in  $\cdot$ NO observed in response to 4 mg/kg SNP.



beyond two treatments. The difference between the human and murine responses is not clear. It may be related to the apparently poorer response in patients exposed to radiotherapy vs. normal controls. We did not expose the human volunteers to more than one treatment in this trial, so we do not know the optimal protocol in normal, healthy humans. An alternative possibility is that there may be differences between mice and humans in bone marrow ·NO synthesis in response to HBO<sub>2</sub>.

There was a discrepancy between the number of CD34-expressing cells and the CFCs observed before the 10th and 20th HBO<sub>2</sub> treatments. Results from CFC experiments conducted with purified monocyte preparations expressing CD34, and those that do not express CD34, indicate that it was the CD34<sup>+</sup> cell population that was responsible for the increase in CFCs in response to HBO<sub>2</sub>. It is not clear why CFCs were not elevated over the initial colony count with cells obtained before the 10th and 20th treatments. The improved growth subsequent to HBO<sub>2</sub> may relate to a small fraction of cells liberated in close proximity to HBO<sub>2</sub> that exhibit improved growth potential. CD34<sup>+</sup> cells mobilized by chemotherapeutic agents and growth factors are reported to exhibit more robust growth potential than older SPCs in the circulation. Cells obtained from patients after they have undergone treatment to mobilize SPCs have twice the plating efficiency (37). Alternatively, we have not ruled out the possibility that recent exposure to HBO<sub>2</sub> may have an antiapoptotic or pro-proliferative effect on SPCs.

Progeny cells from the CFC plates express CXCR4 and VEGFR-2. As CXCR4 is required for progenitor cell homing to sites of injury/ischemia, and VEGFR-2 is present on endothelial progenitors, these findings suggest that some cells mobilized by HBO<sub>2</sub> may function as endothelial progenitors (4, 17). This is also supported by our confocal microscopy findings. In a murine ischemic wound model we have found that HBO<sub>2</sub> stimulates SPCs homing to ischemic wounds, improves vasculogenesis, and improves healing (Goldstein LJ, Gallagher K, Baireddy V, Bauer SM, Bauer RJ, Buerk DG, Thom SR, and Velazquez OC, unpublished observations).

Our study provides new insight into a possible mechanism for HBO<sub>2</sub> therapy. HBO<sub>2</sub> will stimulate neovascularization in humans and in animal models, although mechanisms are poorly understood (15, 24). Others have shown that HBO<sub>2</sub> augments growth factor synthesis (11, 14, 26). If growth factors were elevated in peripheral wounds and sites exposed to radiation, these factors would attract mobilized SPCs to home to the affected areas, where vasculogenesis could occur.

New roles for mobilized SPCs, and also elevations of SCF, are being examined in relation to a number of disorders and clinical interventions (10, 12, 21, 27, 29, 32). A population of CD34<sup>+</sup>/CD133<sup>+</sup> cells have been shown to be pluripotent, capable of repopulating bone marrow in irradiated mice and forming dendritic progenitors (5). These studies offer impetus for further exploration with HBO<sub>2</sub>, given its high degree of safety vs. current methods of SPC mobilization (6, 18, 38). Aural barotrauma occurs in a small number of patients, and rare occurrences of biochemical O<sub>2</sub> toxicity to eyes, lungs, and the central nervous system are virtually always reversible (6, 18, 38). An additional area where SPC mobilization is important is the field of bone marrow transplantation (36). As mobilization of SPCs can be variable in response to chemotherapy, there may be a potential for augmenting the success of

this procedure with concomitant HBO<sub>2</sub>. This issue requires additional investigation.

#### ACKNOWLEDGMENTS

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*Regulation and Function of Stem Cells in the Cardiovascular System*

**Stem cell mobilization by hyperbaric oxygen**

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**ABSTRACT**

We hypothesized that exposure to hyperbaric oxygen (HBO<sub>2</sub>) would mobilize stem/progenitor cells from the bone marrow by a nitric oxide (·NO) -dependent mechanism. The population of CD34<sup>+</sup> cells in the peripheral circulation of humans doubled in response to a single exposure to 2.0 atmospheres absolute (ATA) O<sub>2</sub> for 2 h. Over a course of 20 treatments, circulating CD34<sup>+</sup> cells increased eightfold, although the overall circulating white cell count was not significantly increased. The number of colony-forming cells (CFCs) increased from 16 ± 2 to 26 ± 3 CFCs/100,000 monocytes plated. Elevations in CFCs were entirely due to the CD34<sup>+</sup> subpopulation, but increased cell growth only occurred in samples obtained immediately posttreatment. A high proportion of progeny cells express receptors for vascular endothelial growth factor-2 and for stromal-derived growth factor. In mice,

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HBO<sub>2</sub> increased circulating stem cell factor by 50%, increased the number of circulating cells expressing stem cell antigen-1 and CD34 by 3.4-fold, and doubled the number of CFCs. Bone marrow ·NO concentration increased by 1,008 ± 255 nM in association with HBO<sub>2</sub>. Stem cell mobilization did not occur in knockout mice lacking genes for endothelial ·NO synthase. Moreover, pretreatment of wild-type mice with a ·NO synthase inhibitor prevented the HBO<sub>2</sub>-induced elevation in stem cell factor and circulating stem cells. We conclude that HBO<sub>2</sub> mobilizes stem/progenitor cells by stimulating ·NO synthesis.

nitric oxide; CD34; CD133; CXCR4; cKit; colony-forming cells; progenitor cells

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THE GOAL of this investigation was to determine whether exposure to hyperbaric oxygen (HBO<sub>2</sub>) would mobilize bone marrow-derived stem/progenitor cells (SPCs) in humans and animals. Pluripotent SPCs from adults exhibit properties similar to embryonic SPCs and hold promise for treatment of degenerative and inherited disorders (9, 20). Postnatal neovascularization occurs by sprouting of endothelium from preexisting blood vessels (angiogenesis) and by endothelial SPCs released from the bone marrow that home to foci of ischemia in a process termed vasculogenesis (21). SPC mobilization from the bone marrow can be stimulated by peripheral ischemia, vigorous exercise, chemotherapeutic agents, and hematopoietic growth factors (2, 16, 22, 23, 27, 30, 36). SPCs also can be obtained by direct bone marrow harvesting and ex vivo manipulations (10, 28, 32). Hematopoietic SPCs are typically obtained for the purpose of bone marrow transplantation by administration of chemotherapeutic agents and growth factors (36). Utilizing these agents to obtain autologous SPCs for treating disorders such as organ and limb ischemia, and refractory wounds, has been considered, but application is thwarted because of risks such as acute arterial thrombosis, angina, sepsis, and death (7, 20, 21, 27, 29, 30, 36).

Nitric oxide (·NO) plays a key role in triggering SPC mobilization from the bone marrow via release of the stem cell active cytokine, cKit ligand (stem cell factor, SCF) (1, 8). Because HBO<sub>2</sub> can activate ·NO synthase in different tissues, we hypothesized that exposure to HBO<sub>2</sub> may stimulate SPC mobilization to the peripheral circulation (33, 34). In a murine model, we found HBO<sub>2</sub> augments SPC mobilization and recruitment to ischemic wounds and hastens ischemic wound healing (Goldstein LJ, Gallagher K, Baireddy V, Bauer SM, Bauer RJ, Buerk DG, Thom SR, Velazquez OC, unpublished observations). SPCs have been shown to home to ischemic wounds, where they are required for angiogenesis (3).

HBO<sub>2</sub> therapy is administered for a variety of maladies in a hyperbaric chamber where patients breathe pure O<sub>2</sub> at partial pressures up to 3.0 atmospheres absolute (ATA). HBO<sub>2</sub> is used in a standard fashion as prophylactic treatment to reduce the incidence of osteoradionecrosis (ORN) in patients who must undergo surgery involving tissues previously exposed to radiotherapy (6, 15). We obtained peripheral blood samples from normal human volunteers and from a group of patients undergoing prophylactic HBO<sub>2</sub> in anticipation of surgery to reduce their risk for ORN and examined the blood for the presence of SPCs. We then investigated the mechanism for SPC mobilization in mice. Here, we demonstrate that HBO<sub>2</sub> causes rapid SPC mobilization in both humans and mice and that this occurs via a ·NO-dependent mechanism.

## METHODS

*Stem cell release in humans exposed to HBO<sub>2</sub>.* This protocol was approved by the Institutional Review Board and by the Clinical Trials Scientific Monitoring Committee of the Abramson Cancer Center. Patients are referred to the University of Pennsylvania Institute for Environmental Medicine for prophylactic HBO<sub>2</sub> treatment because of a risk for ORN. A group of these patients was approached, and after informed consent, blood was obtained before and after their first, 10th, and 20th HBO<sub>2</sub> treatment (2.0 ATA O<sub>2</sub> for 2 h). All of these patients had undergone radiotherapy for head or neck tumors; none had open ulcerations, nor were they taking corticosteroids or chemotherapeutic agents. On the basis of current standard of care, they received HBO<sub>2</sub> therapy before undergoing oral surgery due to radiation-induced xerostomia and caries. Men ( $n = 18$ ) had an average age of  $56 \pm 2$  (SE) yr, and women ( $n = 8$ )  $53 \pm 4$  yr. Three inside-chamber paramedic attendants, men with an average age of  $48 \pm 3$  yr, also had blood drawn before and after pressurization to 2.0 ATA for 2 h. These individuals served as a control for the effect of pressure vs. hyperoxia, as they breathe air and not pure oxygen inside the hyperbaric chamber. Three normal, healthy human volunteers, two men and one woman with an average age of  $53 \pm 3$  yr, also underwent 2-h exposures to hyperoxia.

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Citrate anticoagulated blood (16 ml) was centrifuged through Histopaque 1077 (Sigma) at 400 g for 30 min to isolate leukocytes, and cells were washed in PBS. Where indicated, isolated leukocytes underwent further purification to obtain CD34<sup>+</sup> and CD34<sup>-</sup> cells by using paramagnetic polystyrene beads coated with antibody to CD34 (DynaL Biotech, Lake Success, NY). Isolation was carried out exactly as recommended by the manufacturer except that while cells were attached to the beads they were washed only twice, not three times. Normally, the bead selection system achieves 90% purity for CD34-expressing cells but recovers only ~50% of all CD34<sup>+</sup> cells from a cell suspension. Our goal was to assess the growth potential of the CD34<sup>+</sup> and CD34<sup>-</sup> cells separately. With our modified separation method, the aspirated cells that did not attach to the CD34 antibody-coated beads contained only  $1.4 \pm 0.4\%$  (SE,  $n = 9$ ) of the CD34-expressing cells in the total monocyte population, and the recovered cells detached from the beads were  $75 \pm 4\%$  pure. That is, ~25% of the monocytes used in the "CD34<sup>+</sup>" cultures did not express CD34.

For flow cytometry analysis, washed monocytes were suspended in 250  $\mu$ l PBS + 0.5% BSA. Cells were first incubated with rabbit IgG (250  $\mu$ g/ml) for 5 min at 4°C to block Fc receptors and then incubated with a saturating concentration of R-phycoerythrin (RPE)-conjugated mouse anti-human CD34 (Clone 581, a class III CD34 epitope; BD Pharmingen, San Jose, CA), fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CXCR4 (R and D Systems, Minneapolis, MN), and either allophycocyanin (APC)-conjugated mouse anti-human vascular endothelial growth factor-2 receptor (VEGFR-2) (R and D Systems) or APC-conjugated CD133 (Miltenyi Biotec, Auburn, CA) for 30 min at 4°C. Isotype-matched mouse immunoglobulin served as control. Cells were then washed with PBS, and residual erythrocytes were lysed by incubation in 155 mM ammonium chloride, 0.1 mM EDTA and 10 mM sodium carbonate (pH 7.2), centrifuged, and resuspended in PBS. Flow cytometry was performed using a FACScan flow cytometer (Becton Dickinson) at the Abramson Cancer Center Flow Cytometry Core facility. Monocytes were gated on the basis of forward and side laser light scattering, and 100,000 gated cells were analyzed for expression of cell surface markers that may be present on SPCs.

For the colony-forming cell (CFC) assays, monocytes were washed and then suspended in Metho-Cult colony assay medium (StemCell Technologies, Vancouver, BC, Canada), which contains methylcellulose, L-glutamine, fetal bovine serum, bovine serum albumin, recombinant human stem cell factor, granulocyte-monocyte colony stimulating factor, interleukin-3 (IL-3), and erythropoietin. Cultures were initiated with 1 ml of suspension/well of a six-well Petri plate and incubated at 37°C, air with 5% CO<sub>2</sub>, in a fully humidified atmosphere. Nonselected monocytes were cultured at a concentration of 100,000 cells/plate, and isolated CD34<sup>+</sup> cells were cultured at 50,000 cells/plate. Colonies were apparent

and counted using an inverted stage microscope at 14 days.

The phenotype of progeny cells from CFCs plates were analyzed by flow cytometry and confocal microscopy. Cells on CFC plates were harvested by first mixing 5 ml PBS + 0.5 mM EDTA with the semi-soft Metho-Cult medium in plates and then centrifuging at 500 g for 5 min. The cell pellet was washed once in PBS + 0.5% BSA, and one aliquot of cells was characterized by flow cytometry as described above. A second aliquot of cells was resuspended in growth medium and cultured in 24-well plates. Cells were suspended in 60% Dulbecco's modified Eagle's medium (low glucose; GIBCO BRL, Rockford, MD), 40% MCB2-201 medium (Sigma, St. Louis, MO), and the following supplements (all purchased from Sigma): 1x insulin-transferrin-selenium, linolenic acid-BSA,  $10^{-9}$  M dexamethasone,  $10^{-4}$  M ascorbic acid-2-phosphate, 100 U penicillin, and 1,000 U streptomycin. After growth to confluence, cells were scraped from plates, washed in PBS, and spotted onto polylysine-coated microscope slides. Cells were fixed with 1% paraformaldehyde for 10 min and blocked for 1 h at 4°C with Tris-buffered saline (pH 8.3) containing 10 mM Tris, 250 mM NaCl, 0.3% Tween 20, and 1% BSA. Cells were then covered with 50  $\mu$ l 1:1,000 dilution of mouse anti-human von Willebrand factor (BD Pharmingen) made up in PBS + 0.5% BSA for 1 h at 4°C, washed twice with PBS, and then counterstained for 1 h at 4°C with a 1:2,500 dilution of anti-mouse antibody conjugated to Cy3 and FITC-conjugated *Ulex europaeus* agglutinin (Sigma). Cells were examined with a Bio-Rad Radiance 2000 attached to a Nikon TE 300 inverted stage confocal microscope that was operated with a red diode laser at 638 nm and krypton lasers at 488 and 543 nm.

**Mouse studies.** Wild-type and endothelial  $\cdot$ NO synthase knockout (eNOS KO) mice (*Mus musculus*) were purchased (Jackson Laboratories, Bar Harbor, ME), fed a standard rodent diet and water ad libitum, and housed in the animal facilities of the University of Pennsylvania. Mice were exposed to HBO<sub>2</sub> for 90 min following our published protocol (27, 28). In select studies, wild-type mice were pretreated with intraperitoneal N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME), 40 mg/kg, at 2 h before pressurization. Blood was obtained from anesthetized mice [intraperitoneal administration of ketamine (100 mg/kg) and xylazine (10 mg/kg)] by aortic puncture, and bone marrow was harvested by clipping the ends off a femur and flushing the marrow cavity with 1 ml PBS. Leukocytes were isolated in a procedure essentially the same as that described above for human cells, except that blood cells were centrifuged through Histopaque 1083 (Sigma). Antibody staining of cell surface markers was performed as described above by using FITC-conjugated rat anti-mouse stem cell antigen-1 (Sca-1) and RPE-conjugated rat anti-mouse CD34 (both from BD Pharmingen). Mouse stem cell factor was measured using the Quantikine M immunoassay kit from R and D Systems following the manufacturer's instructions.

Bone marrow  $\cdot$ NO level was measured by placing microelectrodes selective for  $\cdot$ NO into the distal femur marrow cavity. Mice were anesthetized, the femurs were exposed, and a 25-gauge needle was used to bore a hole through cortical bone. Nafion-coated  $\cdot$ NO microelectrodes, fabricated from flint glass micropipettes as described in a prior publication (33), were placed within the cavity and held in place by a micromanipulator arm assembly. The mice were then placed within the hyperbaric chamber for exposure to HBO<sub>2</sub>. In selected studies, while breathing just air and not HBO<sub>2</sub>, mice received an intraperitoneal dose of sodium nitroprusside (4–8 mg/kg) to assess whether this manipulation would alter bone marrow  $\cdot$ NO concentration and mobilize SPCs.

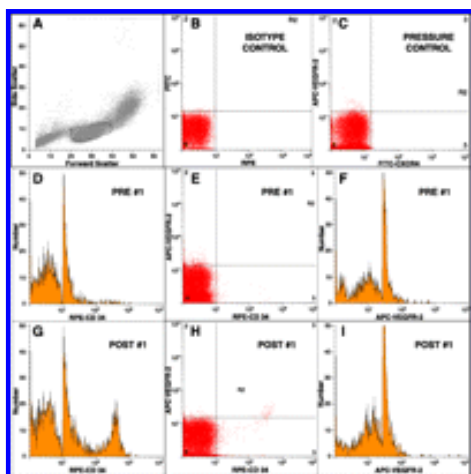
**Statistics.** Statistical analysis of human stem cell numbers was carried out by repeated-measures ANOVA followed by the Dunnett test (SigmaSTAT, Jandel Scientific). CFCs before and after hyperoxia were analyzed by *t*-test, and mouse stem cell mobilization were analyzed by ANOVA followed by Dunn's test. The level of significance was taken as  $P < 0.05$ , and results are expressed as means  $\pm$  SE.

## RESULTS

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*SPCs mobilization in humans.* Blood from patients was obtained before and after the first, 10th, and 20th hyperbaric treatments for ORN prophylaxis (the standard preoperative course of therapy is 20 treatments). Blood leukocytes were harvested and analyzed for the presence of SPCs on the basis of flow cytometry and CFCs.

Results from flow cytometry indicated that there was a range of responses to HBO<sub>2</sub>, and to exhibit this, results from three different patients are shown in [Figs. 1–3](#). [Figure 1A](#) shows a typical scatterdot plot from one cell sample. Before patients were exposed to HBO<sub>2</sub>, very few blood cells were positive for CD34, the most commonly used cell surface marker for SPCs ([20](#)). There were also few cells that expressed VEGFR-2, the receptor for stromal-derived growth factor (CXCR4), or another SPC surface marker, CD133 ([20](#)). These markers were also rarely present on cells from the paramedic attendants inside the hyperbaric chamber, who served as controls for the effect of pressure per se in this study (e.g., [Fig. 1C](#); CD133 data not shown). A comparison of [Fig. 1, D and G](#), shows that the number of cells expressing CD34 was increased in blood after the first HBO<sub>2</sub> treatment. Subsequent to each HBO<sub>2</sub> treatment, we found a small elevation in a population of cells with moderately elevated CD34 expression (exhibiting intensity at between 10 and ~50) and another population with higher intensity of ~100 to 1,000. The dot plots in [Fig. 1, E vs. H](#), show the pattern of CD34 and VEGFR-2 expression for gated cells. [Figure 1H](#) shows a population of cells expressing both surface markers (top right quadrant), and histograms ([Fig. 1, F and I](#)) show the expression of VEGFR-2 on cells before and after the patient's first HBO<sub>2</sub> treatment. In all 26 patients, we found the majority of high-intensity CD34<sup>+</sup> cells also expressed VEGFR-2 at an intensity between 10 and 100.

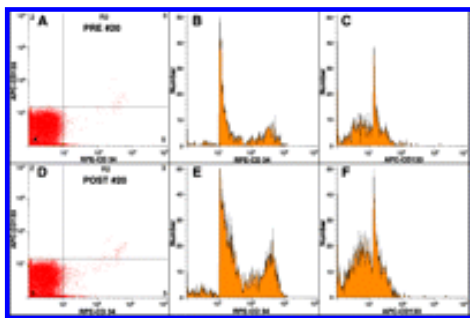


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Fig. 1. Flow cytometry analysis of human leukocytes. Data from a pressure-control subject (paramedic) and a patient before and after their first hyperbaric oxygen (HBO<sub>2</sub>) treatment. *A*: typical forward- and sidescatter dot plot; the black circle indicates the gated cell population analyzed for cell surface markers. *B*: gated sampling of cells incubated with isotype-matched control mouse antibodies conjugated to fluorescein isothiocyanate (FITC) or R-phycoerythrin (RPE). *C*: dot plot for cells from a paramedic, pressure control individual stained for vascular endothelial growth factor-2 receptor (VEGFR-2) and the receptor for stromal-derived growth factor (CXCR4). APC, allophycocyanin. *D–I*: data obtained from 100,000 gated cells from a patient stained for both CD34 and VEGFR-2. The second row of plots (*D–F*) exhibits expression patterns for cells pre-HBO<sub>2</sub>, and the third row of plots (*G–I*) shows the gated cell population post-HBO<sub>2</sub> treatment.



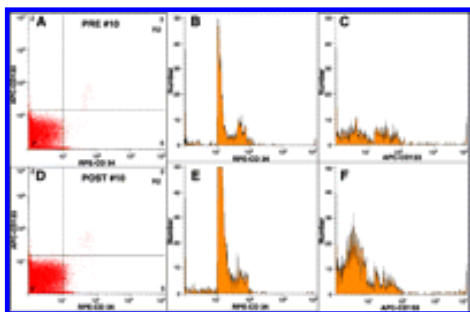
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Fig. 3. Flow cytometry analysis of 100,000 human leukocytes gated as shown in [Fig. 1](#) that were stained for CD34 and CD133. Data are from 1 patient before the 20th HBO<sub>2</sub> treatment (A–C) and after the 20th HBO<sub>2</sub> treatment (D–F).

[Figure 2](#) exhibits responses in a patient before and after his 10th HBO<sub>2</sub> treatment. Cell expression of CD34 was elevated before the 10th treatment, and this will be discussed further below (see [Fig. 4](#)). The CD34<sup>+</sup> population in this patient exhibited somewhat lower surface expression (intensity ~100) than the patients shown in [Figs. 1](#) and [3](#), something we observed in a total of three patients. Circulating endothelial cells express CD34, and they may express VEGFR-2; thus, to more carefully discern whether HBO<sub>2</sub> mobilized SPCs, we also probed cells for expression of CD133 and CXCR4. CD133 is not expressed by endothelial cells, and CXCR4 is expressed on a subset of SPCs ([5](#), [13](#), [17](#), [20](#)). A population of cells expressing both CD34 and CD133 can be seen in [Fig. 2, A and D](#) (top right quadrant before and after the 10th treatment). Histograms for CD34 and CD133 expression on circulating cells are also shown in [Fig. 2](#). [Figure 3](#) shows responses in a third patient before and after the 20th HBO<sub>2</sub> treatment.



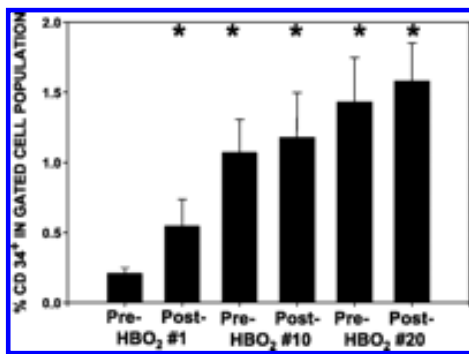
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Fig. 2. Flow cytometry analysis of 100,000 human leukocytes gated as shown in [Fig. 1](#) that were stained for CD34 and CD133. Data are from 1 patient before the 10th HBO<sub>2</sub> treatment (A–C) and after the 10th HBO<sub>2</sub> treatment (D–F).



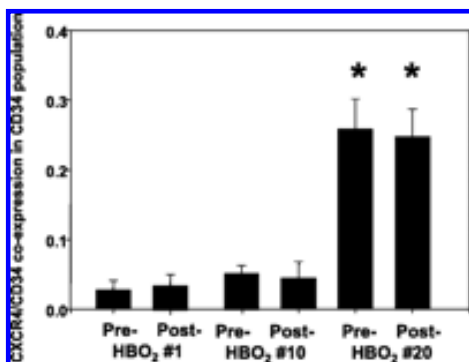


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Fig. 4. Mean CD34<sup>+</sup> population in blood of humans before and after HBO<sub>2</sub> treatments. Data are the fraction of CD34<sup>+</sup> cells within the gated population using leukocytes obtained from 26 patients before and after their 1st, 10th, and 20th HBO<sub>2</sub> treatment. \*Repeated-measures one-way ANOVA,  $P < 0.05$  vs. the pre-HBO<sub>2</sub> first treatment value.

We defined CD34<sup>+</sup> cells as having fluorescence intensity above 10. As shown in [Fig. 4](#), there were persistent elevations in the circulating CD34<sup>+</sup> populations subsequent to the first HBO<sub>2</sub> treatment. However, the number of leukocytes in peripheral blood was not significantly different pre- vs. post-HBO<sub>2</sub> ( $6.8 \pm 0.3 \times 10^3/\mu\text{l}$ , 27% mononuclear preexposure; and  $6.7 \pm 0.8 \times 10^3/\mu\text{l}$ , 28% mononuclear, postexposure), consistent with our previous observations ([35](#)). The fraction of CD34<sup>+</sup> cells in the gated population was  $0.20 \pm 0.05\%$  (SE) before the first HBO<sub>2</sub> treatment and  $1.58 \pm 0.27\%$  after the 20th HBO<sub>2</sub> treatment, an eightfold elevation. SPC mobilization was due to exposure to hyperoxia, and not just pressure, because no augmentation of circulating CD34<sup>+</sup> cells was observed in three paramedic medical attendants who assisted patients inside the hyperbaric chamber (who breathe air, not pure oxygen, while at 2.0 ATA). [Figure 1C](#) shows a cell sample obtained after one paramedic underwent pressurization, and the CD34<sup>+</sup> population looked similar to that shown in [Fig. 1, D and E](#).

The number of CD34-expressing cells increased significantly between the 1st and 10th treatment. There was a trend toward a further increase (not significant) between the 10th and 20th treatment. Although the numbers of CD34<sup>+</sup> cells were not significantly different before vs. after the 10th and 20th treatments, by the 20th treatment the subset of CD34<sup>+</sup> cells that also expressed CXCR4 was significantly higher compared with the dually positive population before HBO<sub>2</sub> started. These results are shown in [Fig. 5](#).



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Fig. 5. Proportion of circulating CD34-expressing cells that also express CXCR4 before and after the 1st, 10th, and 20th HBO<sub>2</sub> treatments ( $n = 26$  patients). \*Repeated-measures one-way ANOVA,  $P < 0.05$  vs. the pre-HBO<sub>2</sub> first treatment value.

Circulating SPCs were also measured in three healthy human subjects before and after a single 2-h exposure to either 1 or 2 ATA O<sub>2</sub>. There was no significant alteration in circulating SPCs due to exposure to 1 ATA O<sub>2</sub> (data not shown), but we found a threefold increase due to exposure to 2 ATA O<sub>2</sub>, a significantly more robust response to a single HBO<sub>2</sub> exposure than observed in the patient population described in Fig. 4. Before exposure to 2.0 ATA O<sub>2</sub>, the mean fraction of CD34<sup>+</sup> cells was  $0.20 \pm 0.02\%$ , and subsequent to hyperoxia at 2 ATA O<sub>2</sub>, the mean fraction of CD34<sup>+</sup> cells was  $0.67 \pm 0.03\%$  ( $P < 0.05$ ).

An alternative approach to assess SPCs was to determine the number of CFCs in peripheral blood. As shown in Fig. 6, CFCs were significantly increased in response to each exposure to HBO<sub>2</sub>. Of note, we did not find elevations in CFCs before the 10th and 20th treatments, although the numbers of CD34<sup>+</sup> cells were elevated (Fig. 4). As these trials were conducted in unselected monocytes, a series of trials was conducted after CD34-expressing monocytes were separated using paramagnetic beads coated with antibody to CD34 (see METHODS). This procedure was carried out on cells from nine patients before and after their 20th HBO<sub>2</sub> treatment. We anticipated better growth in the enriched population, so cells were plated at a reduced density, 50,000 per plate, vs. the 100,000 per plate as in Fig. 6. Before treatment, there were  $12 \pm 1$  colonies/plate, and after HBO<sub>2</sub>,  $23 \pm 2$  colonies grew ( $P < 0.05$ ), whereas in the CD34-negative fraction,  $11 \pm 1$  colonies/plate grew before treatment and  $11 \pm 1$  colonies/plate (no significant difference) grew after HBO<sub>2</sub>.

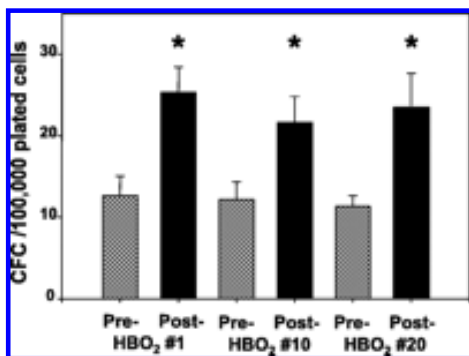


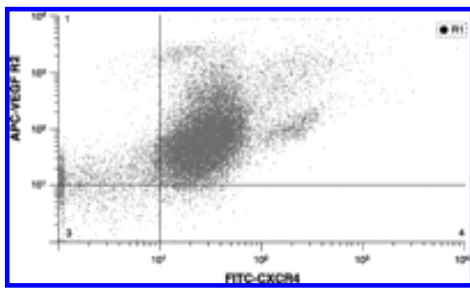
Fig. 6. Colony-forming cells (CFCs) in blood of humans before and after HBO<sub>2</sub> treatments. Data are the colonies counted after a 14-day incubation (all colonies had a myeloid appearance). \*Significant difference by *t*-test performed on each data set pre/post-1st treatment,  $P = 0.036$ ; pre/post-10th treatment,  $P = 0.041$ ; pre/post-20th treatment,  $P = 0.049$ .

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The phenotype of progeny cells from a total of 14 patients was analyzed by flow cytometry. Cells were harvested, and expression of CXCR4 and VEGFR-2 was assessed. Figure 7 shows a typical expression pattern, and we could identify no discernible difference whether cells were cultured after the 1st, 10th, or 20th HBO<sub>2</sub> treatment. Progeny cells were also subcultured and examined by confocal microscopy. Approximately 10% of cells heavily expressed von Willebrand factor and stained positive for *Ulex europaeus* lectin, suggesting that a subset of the mobilized SPCs are endothelial progenitors.



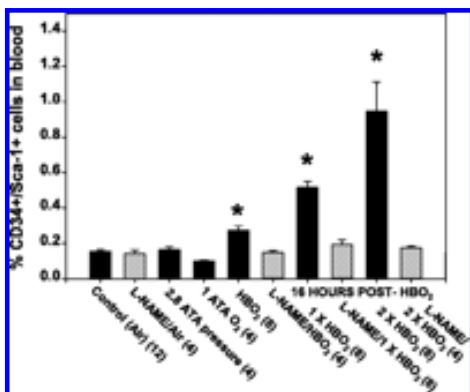
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Fig. 7. Flow cytometry scatterplot of progeny cells obtained from CFC plates from 1 patient after the 10th HBO<sub>2</sub> treatment.

*Studies in mice.* SPCs in peripheral blood of mice were assessed as cells that coexpressed CD34 and Sca-1. In preliminary trials, we found that the most effective pressure for increasing circulating SPCs in mice was 2.8 ATA O<sub>2</sub>. Exposure to 100% O<sub>2</sub> at ambient pressure and exposure to a pressure control, 2.8 ATA pressure by using a gas containing 7.5% O<sub>2</sub> (so that O<sub>2</sub> partial pressure was the same as ambient air, 0.21 ATA O<sub>2</sub>), did not stimulate SPC mobilization (Fig. 8). If leukocytes were harvested immediately after the HBO<sub>2</sub> exposure, there was a significant increase in CD34<sup>+</sup>/Sca-1<sup>+</sup> cells (Fig. 8).



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Fig. 8. Mean CD34<sup>+</sup>/Sca-1<sup>+</sup> cells in blood from mice undergoing HBO<sub>2</sub>. *Left to right:* Control (Air), mice were not exposed to pressure or hyperoxia; L-NAME/Air, mice received 40 mg/kg ip N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME) 3.5 h before cells were harvested; 2.8 ATA pressure, mice were exposed to 2.8 atmospheres absolute (ATA) pressure using a gas containing only 7.5% O<sub>2</sub> (0.21 ATA O<sub>2</sub>) for 90 min before cells were harvested; 1 ATA O<sub>2</sub>, mice were exposed to 100% O<sub>2</sub> at ambient pressure for 90 min before cells were harvested; HBO<sub>2</sub>, mice were exposed to 2.8 ATA O<sub>2</sub> for 90 min, and cells were harvested immediately (within 30–90 min) after depressurization; L-NAME/HBO<sub>2</sub>, mice received 40 mg/kg ip L-NAME 2 h before a 90-min exposure to 2.8 ATA O<sub>2</sub>, and then cells were harvested (in 30–90 min); 16 h post-HBO<sub>2</sub> with 1 x HBO<sub>2</sub>, mice were exposed for 90 min to 2.8 ATA O<sub>2</sub> and then left to breathe air for 16 h before cells were harvested; 16 h post-HBO<sub>2</sub> with L-NAME/1 x HBO<sub>2</sub>, mice received 40 mg/kg ip L-NAME 2 h before a 90-min exposure to 2.8 ATA O<sub>2</sub>, and then cells were harvested 16 h after depressurization; 16 h post-HBO<sub>2</sub> with 2 x HBO<sub>2</sub>, mice received two 90-min exposures to 2.8 ATA O<sub>2</sub> separated by 24 h, and cells were harvested 16 h after the 2nd HBO<sub>2</sub> treatment; 16 h post-HBO<sub>2</sub> with L-NAME/2 x HBO<sub>2</sub>, mice received 40 mg/kg L-NAME ip 2 h before each HBO<sub>2</sub> treatment, and cells were harvested 16 h after the 2nd HBO<sub>2</sub> exposure. All data are means ± SE; *n* = no. of mice in each sample. \**P* < 0.05 vs. the control group (1-way ANOVA).

There is precedent for rapid mobilization of stem cells from bone marrow, but most emigration is believed to occur after a period of cell proliferation within the marrow niche (16, 22). We found that the number of circulating SPCs peaked at 16 h after mice were exposed to 2.8 ATA O<sub>2</sub>, and if mice were exposed to 2.8 ATA O<sub>2</sub> for 90 min on 2 successive days, the number increased even more (Fig. 8). There was no additional increase in peripheral blood SPCs if mice were exposed to more than two HBO<sub>2</sub> treatments. The leukocyte count in peripheral blood and bone marrow did not increase in response to HBO<sub>2</sub>, but there was a significant elevation in CFCs in both blood and bone marrow (Table 1). Soluble kit ligand (stem cell factor, SCF) was significantly elevated in peripheral blood of HBO<sub>2</sub>-exposed mice (Table 1).

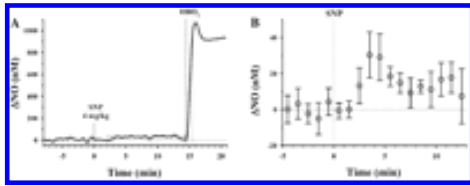
**View this table:** Table 1. *Data from mice*

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A series of studies was carried out to evaluate whether SPC mobilization was a ·NO-mediated response. We found that SPCs were not mobilized in a group of eNOS knockout mice. Air-exposed eNOS knockout mice exhibited more Sca-1/CD34 dual-positive cells in the gated cell population than did wild-type mice,  $0.27 \pm 0.05\%$  ( $n = 4$ ), but there was no evidence of stem cell mobilization in response to HBO<sub>2</sub>. The cell level 16 h after knockout mice were exposed to 2.8 ATA O<sub>2</sub> for 90 min was  $0.26 \pm 0.08\%$  ( $n = 5$ ). We also found that if wild-type mice were injected before HBO<sub>2</sub> with the nonspecific NO synthase inhibitor L-NAME, SPC mobilization did not occur after any of the three different HBO<sub>2</sub>-exposure protocols (Fig. 8). Peripheral blood SCF was not elevated in L-NAME-treated, HBO<sub>2</sub>-exposed mice (Table 1).

Given that ·NO appears to be involved with SPC mobilization, we examined whether administration of a ·NO-donating agent might have an effect similar to HBO<sub>2</sub>. We first examined the alterations in bone marrow ·NO concentrations that resulted from intraperitoneal administration of SNP. We started with a dose of 4 mg/kg, which is anticipated to reduce blood pressure by ~25% for 1 h (31). Figure 9 shows a typical response and also the mean response from three different trials. The ·NO concentration reached a peak of  $39 \pm 10$  nM and returned to baseline by 15 min after SNP injection. Also shown in Fig. 9 is a typical response to HBO<sub>2</sub>, which is consistent with unpublished observations (Goldstein LJ, Gallagher K, Baireddy V, Bauer SM, Bauer RJ, Buerk DG, Thom SR, and Velazquez OC). HBO<sub>2</sub> caused a rapid elevation in bone marrow ·NO that reached a plateau of  $1,008 \pm 255$  nM ( $n = 3$ ), and this level persisted for the duration of the hyperoxic exposure. With the nominal effect of SNP at 4 mg/kg, we carried out four trials using 8 mg/kg, which is approximately three-quarters of the LD<sub>50</sub> for mice (39). Bone marrow ·NO was elevated by  $38 \pm 12$  nM. We also examined circulating SPCs in mice 90 min after SNP administration and found the gated cell Sca-1/CD34 dual-positive population to be  $0.11 \pm 0.02\%$ , insignificantly different from control.



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Fig. 9. Nitric oxide ( $\cdot\text{NO}$ ) concentration in mouse bone marrow. *A*: an example of the elevation in  $\cdot\text{NO}$  after 4 mg/kg ip sodium nitroprusside (SNP). Once the  $\cdot\text{NO}$  level returned to baseline, the mouse was pressurized to 2.8 ATA  $\text{O}_2$ , and the  $\cdot\text{NO}$  level was found to increase markedly. A very similar pattern of responses was observed in 3 separate trials. *B*: average ( $\pm\text{SE}$ ) elevation in  $\cdot\text{NO}$  observed in response to 4 mg/kg SNP.

## DISCUSSION

The results from this study demonstrate that exposure to  $\text{HBO}_2$  will cause rapid mobilization of SPCs in humans, and the number of SPCs remain elevated in blood over the course of 20  $\text{HBO}_2$  treatments. On the basis of the responses in normal human controls, it appears that previous exposure to radiation diminishes the response to one  $\text{HBO}_2$  treatment. Radiotherapy is known to reduce the mobilization that occurs in response to chemotherapeutic agents and growth factors (19, 25).

Studies in mice indicate that  $\text{HBO}_2$  stimulates SPCs mobilization, although the dose response may differ from that observed in humans. We did not systematically examine the time course or dose response for SPC mobilization by  $\text{HBO}_2$  in humans. A typical pattern for scientific/medical discovery is to carry out studies in model systems and then verify their occurrence in human beings. In our study, we made our initial observations of SPC mobilization by  $\text{HBO}_2$  in humans and then investigated the responses in animals to elucidate the mechanism. In fact, had we started our work with animals, we may not have made our discovery because the  $\text{HBO}_2$  protocol typically used (2.0 ATA  $\text{O}_2$  for 1–2 h) causes only a rather nominal effect in mice. We found that the most effective pressure for increasing circulating SPCs in mice was 2.8 ATA  $\text{O}_2$ .

Observations with eNOS knockout mice and the inhibitory effect of L-NAME in wild-type mice indicate that  $\text{HBO}_2$  mobilized SPCs by a  $\cdot\text{NO}$ -dependent mechanism.  $\text{HBO}_2$  elevates SCF in peripheral blood, and this too is inhibited by L-NAME. These findings are consistent with published work showing that stimulation of bone marrow  $\cdot\text{NO}$  synthase will activate metalloproteinase-9 to cleave SCF from its membrane linkage, thus allowing for SCF-mediated SPCs mobilization (1, 8).  $\text{HBO}_2$  causes a significant elevation in bone marrow  $\cdot\text{NO}$  concentration that cannot be replicated with infusion of SNP.

In patients, there was a significant increase in numbers of  $\text{CD}34^+$  cells between the 1st and 10th hyperbaric treatment. In mice, we found that two treatments yielded significantly greater mobilization than one, but no further increase occurred beyond two treatments. The difference between the human and murine responses is not clear. It may be related to the apparently poorer response in patients exposed to radiotherapy vs. normal controls. We did not expose the human volunteers to more than one treatment in this trial, so we do not know the optimal protocol in normal, healthy humans. An alternative possibility is that there may be differences between mice and humans in bone marrow  $\cdot\text{NO}$  synthesis in response to  $\text{HBO}_2$ .

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There was a discrepancy between the number of CD34-expressing cells and the CFCs observed before the 10th and 20th HBO<sub>2</sub> treatments. Results from CFC experiments conducted with purified monocyte preparations expressing CD34, and those that do not express CD34, indicate that it was the CD34<sup>+</sup> cell population that was responsible for the increase in CFCs in response to HBO<sub>2</sub>. It is not clear why CFCs were not elevated over the initial colony count with cells obtained before the 10th and 20th treatments. The improved growth subsequent to HBO<sub>2</sub> may relate to a small fraction of cells liberated in close proximity to HBO<sub>2</sub> that exhibit improved growth potential. CD34<sup>+</sup> cells mobilized by chemotherapeutic agents and growth factors are reported to exhibit more robust growth potential than older SPCs in the circulation. Cells obtained from patients after they have undergone treatment to mobilize SPCs have twice the plating efficiency (37). Alternatively, we have not ruled out the possibility that recent exposure to HBO<sub>2</sub> may have an antiapoptotic or pro-proliferative effect on SPCs.

Progeny cells from the CFC plates express CXCR4 and VEGFR-2. As CXCR4 is required for progenitor cell homing to sites of injury/ischemia, and VEGFR-2 is present on endothelial progenitors, these findings suggest that some cells mobilized by HBO<sub>2</sub> may function as endothelial progenitors (4, 17). This is also supported by our confocal microscopy findings. In a murine ischemic wound model we have found that HBO<sub>2</sub> stimulates SPCs homing to ischemic wounds, improves vasculogenesis, and improves healing (Goldstein LJ, Gallagher K, Baireddy V, Bauer SM, Bauer RJ, Buerk DG, Thom SR, and Velazquez OC, unpublished observations).

Our study provides new insight into a possible mechanism for HBO<sub>2</sub> therapy. HBO<sub>2</sub> will stimulate neovascularization in humans and in animal models, although mechanisms are poorly understood (15, 24). Others have shown that HBO<sub>2</sub> augments growth factor synthesis (11, 14, 26). If growth factors were elevated in peripheral wounds and sites exposed to radiation, these factors would attract mobilized SPCs to home to the affected areas, where vasculogenesis could occur.

New roles for mobilized SPCs, and also elevations of SCF, are being examined in relation to a number of disorders and clinical interventions (10, 12, 21, 27, 29, 32). A population of CD34<sup>+</sup>/CD133<sup>+</sup> cells have been shown to be pluripotent, capable of repopulating bone marrow in irradiated mice and forming dendritic progenitors (5). These studies offer impetus for further exploration with HBO<sub>2</sub>, given its high degree of safety vs. current methods of SPC mobilization (6, 18, 38). Aural barotrauma occurs in a small number of patients, and rare occurrences of biochemical O<sub>2</sub> toxicity to eyes, lungs, and the central nervous system are virtually always reversible (6, 18, 38). An additional area where SPC mobilization is important is the field of bone marrow transplantation (36). As mobilization of SPCs can be variable in response to chemotherapy, there may be a potential for augmenting the success of this procedure with concomitant HBO<sub>2</sub>. This issue requires additional investigation.

## GRANTS

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## FOOTNOTES

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