ORIGINAL ARTICLE

Effect of Hyperbaric Oxygen on the Growth Factor Profile of Fibroblasts

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Objectives: Hyperbaric oxygen (HBO) has been used in the clinical setting to heal problem wounds, yet its direct effects on fibroblasts are not clear. The present study evaluates the effects of HBO on the growth and autocrine production of growth factors by fibroblasts grown in an in vitro, serum-free environment.

Methods: Human dermal fibroblasts were propagated in serum-free media and subjected to daily 90-minute HBO treatments at 1.0, 1.5, 2.0, 2.5, and 3.0 atm of pressure for 7 consecutive days. Cell proliferation and growth-factor assays for basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and transforming growth factor β1 (TGF-β1) were performed on days 1, 3, 5, and 7.

Results: On day 1, HBO inhibited growth of fibroblasts at all atmospheric pressures compared with control. By day 7, cell proliferation was significantly enhanced only in cells treated with 2.0-atm HBO compared with controls. Secretion of bFGF was significantly increased by HBO-treated fibroblasts on day 1; VEGF levels slightly increased with HBO treatment on day 1, but this effect was not statistically significant; TGF-β1 levels were detectable on day 1 only for control and HBO-treated cells at 1.0 atm, and not detectable for any cell groups after day 1.

Conclusions: These results suggest that daily HBO treatment enhances the growth of fibroblasts when administered to a critical degree. Also, HBO appears to directly effect fibroblast production of autocrine growth factors on initial exposure. We postulate that fibroblasts possess the ability to respond to hyperoxia directly, which causes changes in cell signaling pathways involved in cellular proliferation and growth factor production.

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HYPERBARIC OXYGEN (HBO) therapy is the administration of 100% oxygen at atmospheric pressures greater than that at sea level, thus increasing dissolved oxygen content to above physiologic levels. Treatment with HBO has been available and advocated for many years as a therapeutic modality for carbon monoxide poisoning, decompression sickness, arterial gas embolism, osteomyelitis, severe anemia, and wound healing.[1,2]

In the clinical setting of problem wounds, HBO has been advocated in several studies to reduce wound dehiscence, infections, and delayed healing, though strong data from blinded, prospective studies are lacking.[2,3] Presumably, HBO treatment exerts its effect by raising available oxygen to levels favorable for wound healing. Most of the research in HBO therapy has been done in vivo. In 1972, Pai and Hunt[4] observed that collagen deposition—the process that fills tissue defects and supports new blood vessels—proceeds in proportion to tissue oxygen levels. Scott,[5] in his study of the effect of oxygen on connective tissues, observed that oxygen accelerates the synthesis of glycosaminoglycans. While there is evidence to suggest that intermittent exposure to HBO may play a direct role in fibroblast collagen synthesis and vascular growth, the autocrine processes involved are clearly not fully understood.

There have been few in vitro studies examining the effect of HBO on cells in culture. Dimitrijevich et al[6] studied the effect of HBO on human skin cells in culture and in human dermal and skin equivalents. In that study, normal human dermal fibroblasts, keratinocytes, melanocytes, dermal equivalents, and skin equivalents were exposed to HBO at pressures up to 3 atm for up to 10 consecutive daily treatments lasting 90 minutes each. An increase in fibroblast proliferation, collagen production, and keratinocyte differentiation was observed. A study by Roberts and Hard-

ing[7] showed that while production of glycosaminoglycans increased in cultured fibroblasts exposed to HBO, fibroblast proliferation actually decreased by 7%.

Until now, there has been no study on the effect of HBO on fibroblasts cultured in a serum-free environment. Since the only growth factors in serum-free systems are the products of the cells themselves, assaying for autocrine products can be achieved without exogenous contributions. Serum-free models have already been established for keloid fibroblasts and used successfully to test pulsed carbon dioxide laser energy as a wound healing modulator.[8,9] Recently, a serum-free model has been developed for growing irradiated fibroblasts on commercially available media.[10] The purpose of the present study was to characterize the growth of fibroblasts exposed to HBO as well as to quantify the production of 3 specific growth factors involved in wound healing during HBO treatment: basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and transforming growth factor β1 (TGF-β1). By examining the cellular effects of HBO, we may elucidate the mechanisms behind its therapeutic use in patients with problem wounds.

METHODS

FIBROBLAST PRIMARY CULTURES

Fibroblast primary cell lines were established from skin obtained from operative specimens from the eyelids of a 48-year-old white man undergoing blepharoplasty. Approval to use operative specimens that would otherwise be discarded was obtained in advance from the human subjects committee of Stanford University. Using sterile technique under a laminar flow hood, the dermal specimens were minced into approximately 1-mm^3 fragments on a Petri dish with a sterile scalpel blade, then washed in Dulbecco phosphate-buffered saline solution with 5% penicillin/streptomycin/amphotericin (PSA) (Gibco, Grand Island, NY). The specimens were then placed in scored 75-cm^2 tissue culture flasks (T75; Falcon, Becton-Dickinson, Franklin Lakes, NJ) with 10 mL of primary culture medium (10% fetal bovine serum in Dulbecco modified Eagle media with 1% L-glutamine and 1% PSA) (Gibco). The flasks were then stored in a humidified incubator at 37°C with a 5% carbon dioxide atmosphere.

After 24 hours, the medium was changed with 10 mL of primary culture medium, then changed every 2 to 4 days until fibroblasts were visualized under a light microscope to be growing outward from the explanted tissue. At that time, the tissue was removed and cells subcultured into 75-cm^2 culture flasks. Primary culture medium was changed every third to fourth day. Successive cultures were passed at confluence.

CELL PLATING IN SERUM-FREE MEDIUM

Confluent cells from the second passage were released from the flask wall using 0.05% trypsin. The trypsin was then inactivated using trypsin soybean inhibitor (Gibco) in a 1:1 ratio. Cells were then suspended in UltraCULTURE (Biowhittaker, Walkersville, Md) serum-free medium and counted in duplicate using a hemocytometer. Cell viability was confirmed with trypan blue dye exclusion. Fibroblasts were then seeded in UltraCULTURE at a density of 5×10^3 cells in each well of a 96-well plate (Falcon, Becton-Dickinson), with a standard volume of 100 µL per well.

HBO ADMINISTRATION

Fibroblast cultures within the 96-well plates were transferred to an HBO chamber (Biotyme, Whittier, Calif), which was sealed, flushed with 100% oxygen, then pressurized to the appropriate level as measured by an attached barometer. Treatment groups were exposed to 100% oxygen at 1.0, 1.5, 2.0, 2.5, or 3.0 atm daily for 90 minutes for 7 consecutive days. Control plates were exposed to ambient room air in a sterile hood for 90 minutes daily for the duration of the experiment. Following treatment exposures, the chamber was decompressed and cell culture plates promptly returned to the incubator. Cell-free supernatant for all groups was collected at days 1, 3, 5, and 7 and stored in microcentrifuge tubes at –20°C for use in later growth factor assays.

CELL COUNTING AND GROWTH CURVE GENERATION

Cell counts were performed using the WST-1 assay (Boehringer Mannheim, Indianapolis, Ind) at 1, 3, 5, and 7 days after initiation for growth curve generation. The WST-1 assay is a colorimetric assay used in the quantification of cell proliferation and cell viability based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells. Assays were read using an automated plate reader (ELx800; Bio-Tek Instruments Inc, Winooski, Vt). Optical densities were analyzed with KC4 software (Bio-Tek Instruments Inc), and cell counts determined by comparison with a standard curve generated from known cell quantities.

GROWTH FACTOR ASSAYS

Expression of bFGF, VEGF, and TGF-β1 for all groups was measured using solid-phase enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, Minn) from supernatant collected at 1, 3, 5, and 7 days after initiation of treatment. Assays were performed in triplicate and read using an automated plate reader; optical densities were analyzed with KC4 software. Growth factor quantities were determined by comparison with standard curves generated with each ELISA performed.

STATISTICAL ANALYSIS

Cell counts from experimental and control plates at each measurement point were expressed as a mean from 12 replicate cell wells. Differences between HBO-treated and control cell plates were then compared by paired *t* test and considered significantly different at $P<.05$. Growth factor quantities generated from ELISA tests were converted to units per cell, and mean values were compared with control.

RESULTS

All fibroblast cultures were sustained successfully in serum-free media for the duration of the experiment. Control cells (those in ambient room pressure and oxygen content) demonstrated roughly linear growth, doubling in number over the 7 days. At day 1, all HBO-treated cells initially showed a relative decrease in proliferation compared with controls, though this finding was only statistically significant for the 2.0- and 2.5-atm groups ($P<.05$). On day 3, only the 3.0-atm group demonstrated significantly decreased proliferation compared with controls ($P<.05$); all other groups showed no difference com-

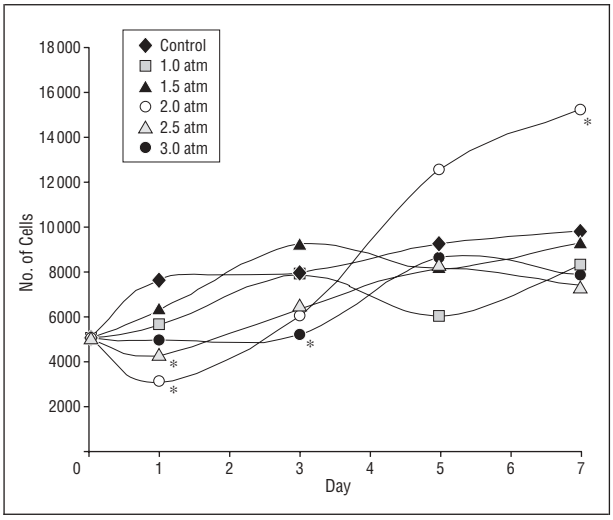

Figure 1. Mean growth curves of fibroblasts exposed to varying levels of daily 90-minute hyperbaric oxygen (HBO) treatment. Control cells were exposed to ambient room pressure and oxygen content. All HBO-treated cells demonstrated less growth than controls at day 1. Only treatment to 2.0 atm resulted in an increased number of cells by day 7. Each asterisk indicates $P<.05$ compared with the control group.

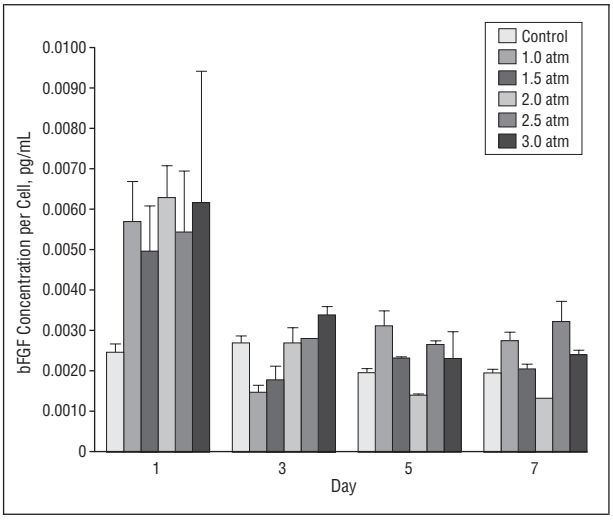

Figure 2. Mean basic fibroblast growth factor (bFGF) levels measured in serum-free supernatants collected from human dermal fibroblast cell cultures exposed to varying levels of daily hyperbaric oxygen treatment. Error bars indicate SD.

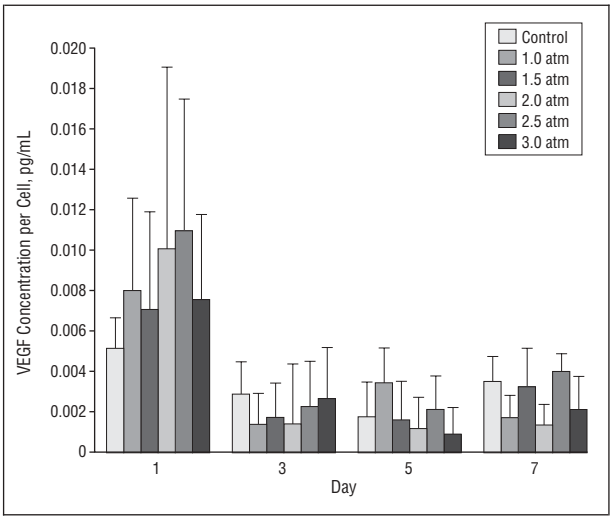

Figure 3. Mean vascular endothelial growth factor (VEGF) levels measured in serum-free supernatants collected from human dermal fibroblast cell cultures exposed to varying levels of daily hyperbaric oxygen treatment. Error bars indicate SD.

pared with controls. After day 3, HBO-treated cell proliferation was no different than controls except for the 2.0-atm group, which showed a marked relative increase in proliferation after day 3 that became statistically significant by day 7 ($P<.01$) (**Figure 1**).

Growth factor assays showed an increase in production of bFGF in all HBO-treated cells at day 1 (**Figure 2**); this increase was statistically significant for all experimental groups ($P<.05$) except for the 3.0-atm group. However, this increase in bFGF secretion by HBO-treated fibroblasts diminished by day 3 and thereafter paralleled the production of control cells, which stayed fairly constant over the measured period.

A similar profile was seen with VEGF: all HBO groups secreted more of this growth factor than the control group did on day 1, although this difference was not statistically significant. After day 1, there were no differences in VEGF production between HBO-treated cells and controls (**Figure 3**).

A low amount of TGF-β_1 was detected in the control group (2.66×10^{-4} pg/mL per cell) and in the 1.0 atm–treated cells (4.63×10^{-4} pg/mL per cell) at day 1, and thereafter this growth factor was not detectable by ELISA in any of the cell groups (data not shown).

COMMENT

The therapeutic administration of HBO was used as early as 1873 for the successful treatment of decompression sickness in miners.[11] Since then, HBO has proven to be effective treatment for gas embolism and carbon monoxide poisoning, largely based on the increased dissolved oxygen content in serum that occurs with HBO treatment. The use of HBO as a modulator of wound healing, however, has been hindered by a lack of understanding of its direct cellular effects. Treatment with HBO is not without morbidity: the clinical use of HBO has been associated with complications of seizure, stroke, myocardial infarction, pulmonary toxic effects, eustachian tube dysfunction, and ocular problems.[12,13]

Previous in vitro studies looking at the cellular effects of HBO in wound healing have exposed a complex picture. Hyperoxia appears to enhance bactericidal activity, either directly or via modulation of white blood cell function.[2] Treatment with HBO has been shown to increase tissue vascularity, presumably by creating a favorable oxygen gradient for proangiogenic processes to act on endothelial cells. For fibroblasts, adequate oxygen levels are known to be required for hydroxylation processes involved in collagen release[14]; hyperoxic conditions appear to enhance collagen and glycosaminoglycan synthesis.[7,15]

In our study, fibroblast cell proliferation in response to HBO was initially inhibited but then recovered after repeated daily treatments. This finding sug-

gests a delayed cellular adaptation to the HBO treatments. One possible explanation for this is the stimulation of hyperoxia-induced cellular pathways leading to adaptive protection from oxygen free-radical damage. Speit et al[16] demonstrated that lymphocytes of HBO-treated patients show evidence of DNA damage only after the first treatment; subsequent treatments fail to result in DNA damage. This finding they attribute to an up-regulation of expression of the antioxidant enzyme heme oxygenase-1 found 24 hours after initial HBO treatment. Perhaps a similar phenomenon involving oxygen radicals would explain the initial inhibition of growth and subsequent recovery of fibroblasts in our study. Ongoing experiments in our laboratory involve addition of exogenous oxygen free-radical scavengers to fibroblast cell culture media to investigate whether the initial HBO-induced inhibition of cell proliferation can be prevented.

Our finding that fibroblast cell proliferation by day 7 is enhanced with HBO treatment at 2.0 atm but not with higher or lower levels of HBO suggests that there is a critical level of hyperoxia required to stimulate fibroblast cell proliferation. Interestingly, this finding of a "right amount" of HBO exposure has been reported in prior studies. Zhao et al[17] reported increased new granulation tissue and vascularity in a rabbit ear wound model in animals treated with HBO at 2 atm, but not at 1 atm. Tompach et al[14] showed that whereas measured proliferation of fibroblasts exposed to 2.4-atm HBO was significantly greater than that of controls, exposure to 4-atm HBO resulted in cellular activity of only 79% of the control value. Dimitrijevich et al[6] noted that the effect of increased growth of cultured fibroblasts seen after daily HBO treatments at 1 and 2.5 atm was not observed with 3-atm treatment. It is likely that too-high levels of HBO negatively affect fibroblast proliferation, whether from oxygen toxic effects or from mechanically induced effects of high atmospheric pressure.

The wound healing process involves complex interactions of locally produced growth factors. It has been shown that addition of exogenous growth factors acts synergistically to induce wound healing when used in conjunction with HBO.[17] Until now, examination of the local production of specific growth factors by fibroblasts exposed to HBO has not been performed. The serum-free model used in the present study was successful in supporting cultured fibroblasts and allowed for the quantification of bFGF, VEGF, and TGF-β1.

Basic fibroblast growth factor is a single polypeptide produced by multiple cells including dermal fibroblasts; it is mitogenic, promotes cell survival, inhibits collagen production, and stabilizes cellular phenotype.[9] The results of the present study clearly showed an increased secretion of bFGF in cells after initial HBO exposure. Repeated exposure, however, does not appear to maintain elevated bFGF levels in the cell supernatant, suggesting a possible cellular adaptation to the hyperoxic enviroment.

Vascular endothelial growth factor is a potent stimulator of angiogenesis.[18] While stimulation of VEGF production has been linked to hypoxic conditions, recent evidence suggests that intermittent hyperoxia from HBO treatment may also increase VEGF levels in the setting of wound healing. Marx et al[18] showed in irradiated tissue that increased vascularity was induced with daily 2.4-atm HBO treatment, but not with 1-atm treatments. Sheikh et al[19] demonstrated in a rat wound model that levels of VEGF are significantly increased with exposure to intermittent 2.1-atm HBO. In the present study, there was a trend of increased VEGF production with initial HBO treatment, although there were no statistically significant differences between HBO cells and controls, suggesting that hyperoxia-induced changes in VEGF levels are not primarily a result of altered production by dermal fibroblasts.

Transforming growth factor β1, 1 of 3 human isoforms, stimulates the production of collagen, inhibits protease production, and enhances mitogenesis.[9] Production of TGF-β1 is increased in keloid fibroblasts[20] and also increased in fetal fibroblasts compared with adult fibroblasts.[21] Our study revealed detectable levels of this growth factor only in control cells and 1.0-atm HBO cells at day 1, suggesting that TGF-β1 production was effectively inhibited in cells treated with HBO greater than 1.0 atm. This result points to an inverse relationship between oxygen level and production of this growth factor—a relationship supported by a finding by Falanga et al[22] that up-regulation of TGF-β1 messenger RNA synthesis occurs in human dermal fibroblasts exposed to hypoxic conditions.

Although wound healing is a dynamic process involving complex interactions among a variety of cells, results from the present study reveal that HBO acts as a direct modulator of fibroblast proliferation and regulator of growth factor production. We agree with Davidson and Mustoe[23] that oxygen in wound healing is "more than a nutrient" and assert that fibroblasts have the ability to respond to hyperoxic conditions with specific cellular signaling pathways. In current and future experiments in our laboratory, we hope to further elucidate these pathways to better understand the cellular effects of HBO as a wound healing modulator.

In conclusion, (1) fibroblasts in culture exposed to HBO treatment experience a transient initial decrease in cell proliferation. (2) Daily HBO treatment at 2.0 atm selectively stimulates fibroblast proliferation after 7 days; lower or higher levels of HBO do not appear to have this effect. (3) HBO produces a transient increase in bFGF production by fibroblasts and may inhibit TGF-β1 production. Production of VEGF by fibroblasts is not significantly affected by HBO treatment. (4) Serum-free medium continues to provide a means to sustain viable fibroblast cells in culture and quantify production of endogenous growth factors.

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