Dose-dependent hyperbaric oxygen stimulation of human fibroblast proliferation

KARIN HEHENBERGER°; KERSTIN BRISMAR, MD, PhD°; FOLKE LIND, MD, PhD°; GUNNAR KRATZ, MD, PhD°

Diabetic wounds are characterized by a prolonged wound healing process with insufficient formation of granulation tissue. Systemic hyperbaric oxygen therapy has been observed to improve the healing of these wounds. However, the mechanism(s) responsible for these findings are not yet fully elucidated. In the present study we have studied the in vitro effects of hyperbaric oxygen on proliferation of human fibroblasts from normal skin and from chronic foot ulcers in non-insulin-dependent diabetics. A 1-hour exposure to hyperbaric oxygen at oxygen pressures between 106 and 300 kPa (795 to 2250 mm Hg) increased the proliferation in both diabetic and normal fibroblasts. The stimulatory effect was dose-dependent, with a peak increase in cell proliferation at 250 kPa and 200 kPa for normal and diabetic cells, respectively. The effects were not due to hydrostatic pressure per se. These results suggest that hyperbaric oxygen could stimulate fibroblast activity in the diabetic wound, a finding that could explain the enhanced formation of granulation tissue seen clinically in wounds treated with hyperbaric oxygen. We also speculate that mechanisms other than just increased oxygen availability may be responsible for our findings. (WOUND REP REG 1997;5:147-50)

Wound healing involves several cell types, fibroblasts being among the most important for granulation tissue formation. In normal undamaged tissue, fibroblasts are sparsely distributed throughout the connective tissue matrix and generally quiescent. After injury, fibroblasts are activated to migrate from adjacent tissue into the wound site where they proliferate and produce collagen, elastin, and proteoglycans, which will reconstruct the connective tissue.

One characteristic of chronic diabetic wounds is insufficient formation of granulation tissue, which could be due to impaired fibroblast activity. In animals with acute experimental diabetes, the early inflammatory response after wounding is impaired and fibroblast proliferation, as well as endothelial cell proliferation, are reduced, as is the accumulation of collagen. Fibroblasts cannot synthesize collagen in the absence of molecular oxygen, which is required for the hydroxylation of proline and lysine residues in the nascent procollagen molecule. In diabetics, microangiopathy and/or macroangiopathy together with increased glycosylated hemoglobin lead to decreased oxygenation of the tissue. During conditions with increased oxygen demands, such as wound healing, the impaired oxygenation will be even more severe. For example, injuries damage capillaries and lead to an accumulation of inflammatory cells which consume oxygen at the wound site.

Inadequate oxygenation is said to be the most frequent common cause of any nonhealing wound. In line with this concept, an increase in oxygen tension (PO₂) has been reported to improve wound healing. Thus, in
nondiabetic animal models, it has been shown that fibroblast collagen synthesis increases above normal with hyperbaric oxygen (HBO) conditions (i.e., oxygen tensions greater than normal ambient pressure >1 bar absolute or 100 kPa).9

Systemic HBO therapy has been shown to elevate the PO2 tension in both normal and hypoxic tissues, and it has been used in the treatment of chronic, nonhealing, ischemic wounds.6,10-12 However, the underlying mechanism(s), with regard to their therapeutic effect, are not fully understood.

The aim of this investigation was to study the in vitro effects of short-term treatment with various doses of HBO on the proliferation of human fibroblasts derived from normal skin and from chronic diabetic wounds.

MATERIALS AND METHODS

Materials

Analytic reagents were obtained from Sigma Chemical Co. (St. Louis, Mo.), Hoechst 33258 dye was obtained from Polysciences Ltd. (Goar, Germany), and cell culture materials were obtained from Gibco (Paisley, United Kingdom).

Establishment of human cell cultures

Normal human dermal fibroblasts were obtained from biopsy samples taken from patients undergoing mammary reduction plastic surgery. Diabetic fibroblasts were obtained from biopsy samples from chronic diabetic ulcers (duration more than 2 months) during wound revision in five patients with non-insulin-dependent diabetes mellitus. The state of the diabetic ulcers were nonischemic neuropathic plantar foot ulcers. The biopsy samples were taken with a biopsy punch (4 mm diameter, 4 to 5 mm deep) from the granulation tissue close to the wound margin. Fibroblasts were obtained from the tissues by explant techniques in 12-well plates. The medium used was Dulbecco’s modified Eagle’s medium containing 10% newborn calf serum, penicillin 50 IU/ml, streptomycin 50 μg/ml, and L-glutamine 2 mmol/L. Cells of the fifth to eighth generation were used in the study.

Informed consent was obtained from each patient, and approval was received from the local ethics committee for research in human subjects in accordance with the declaration of Helsinki.

Experimental design

Fibroblasts were seeded into 12-well tissue culture plates and allowed to adhere for 24 hours. Serum was removed from the culture media for the next 24 hours, at which time the experiments were conducted.

Cells from diabetic and normal tissue were placed in hyperbaric chambers (Monoplace hyperbaric chamber, model 2500 B; Sechrist Industries, Inc., Anaheim, Calif.) without covering the wells with the lids. The cells were treated with air (21% O2) or oxygen (100% O2) for 1 hour at different pressures ranging from 106 to 300 kPa (795 to 2250 mm Hg). Cells treated with air at a pressure of 106 kPa (PO2 = 21 kPa) were used as controls, and cells treated with air at 250 kPa (PO2 = 52 kPa) were used to exclude the possible effects of hydrostatic pressure, per se, by comparison with cells treated with oxygen at 250 kPa (PO2 = 250 kPa). Hyperbaric PO2 levels were obtained by 1-hour treatments with oxygen at chamber pressures of 106, 150, 200, 250, and 300 kPa.

Total cell number was measured immediately before and 24 hours after the 1-hour HBO treatment by a fluorometric DNA assay in which one assumes DNA content is proportional to cell number. We have modified a method for measuring cell number that is based on the binding of Hoechst 33258 dye to DNA.13 Five hundred microliters of a trypsin (0.5 gm/L)-ethylenediamine tetraacetic acid (0.2 gm/L) was used to remove the cells. An equal volume (500 μl) of 0.01% Triton X-100 in TNE (Tris-Cl 10 mmol/L, NaCl 100 mmol/L, EDTA 10 mmol/L, pH 7.0) was then added. Cell lysis was verified by microscopy. Two milliliters of 0.1 μg/ml Hoechst dye in TNE was combined with the solubilized cells, and the fluorescence of the samples was measured with an excitation wavelength of 365 nm and an emission wavelength of 458 nm in a TKO 100 mini-fluorometer (Hoefer Scientific, San Francisco, Calif.). The fluorometer was calibrated with a standard solution of calf thymus DNA.

Statistics

Differences between groups were analyzed with Student’s t-test for two independent samples, whereas differences within the groups at various oxygen pressures were analyzed with Student’s t-test for paired samples. Experiments were done in triplicate with five different samples.

RESULTS

The cell number of the diabetic fibroblasts, as determined by DNA content, was significantly lower (p < 0.01) compared with normal fibroblasts (75% ± 11%) after 24 hours of serum starvation and immediately before the oxygen exposure.

Measurement of DNA content 24 hours after a 1-hour exposure to HBO showed that both normal and diabetic fibroblasts increased their total cell number compared with untreated cells (i.e., cells at 21 kPa PO2). The increment was found to be dose-dependent (Figure 1). At a PO2 of 106 kPa (795 mm Hg) the normal fibroblasts had a significantly higher (p < 0.05) cell number (12% ± 5%) compared with the untreated cells. Similarly, but more pronounced, the diabetic fibroblasts increased their total cell number by 36% ± 7% (p < 0.01) as compared with untreated cells. The maximum cell numbers, and hence peak proliferation rates, were reached at a PO2 of 250 kPa.
Figure 1 DNA content, expressed as fluorescence units, of human fibroblasts derived from normal skin (filled bars) and from chronic diabetic wounds (shaded bars) 24 hours after a 1-hour treatment at multiple oxygen pressures. Values represent the mean ± standard deviation of five different patients, each assayed in triplicate. Significant difference (*p < 0.05, **p < 0.01) compared with 21 kPa (untreated cells). (1875 mm Hg) in the normal and 200 kPa (1440 mm Hg) in the diabetic cells, corresponding to an increase of 43% ± 10% (p < 0.01) in normal and 63% ± 11% (p < 0.01) in diabetic cells (Figure 2).

At 200 kPa level of oxygen, the fibroblasts derived from chronic diabetic wounds had reached the same total cell number as untreated normal fibroblasts derived from uninjured skin. When comparing the experiments with air and oxygen at 250 kPa, there was a significant difference that correlated with the difference in O₂ pressures (52 kPa versus 250 kPa) (Figure 1).

DISCUSSION
In the present study we show that fibroblasts from chronic diabetic wounds have a lower proliferation rate compared with those from normal uninjured skin. This difference can be abolished by a 1-hour HBO treatment of the diabetic fibroblasts. Both normal and diabetic fibroblasts show a dose-dependent increase in cellular proliferation after treatment with HBO at 106 kPa (795 mm Hg) and above. The experiments with either air or oxygen at 250 kPa indicate that hydrostatic pressure, per se, did not contribute to our findings of a dose-dependent HBO stimulation of human fibroblast proliferation.

Clinically, HBO used in the treatment of chronic wounds has been reported to increase collagen deposition and granulation tissue formation. It has been shown that impaired metabolic control in diabetes mimics hypoxic conditions resulting in an increased ratio of reduced nicotinamide adenine dinucleotide (NAD) to oxidized NAD. This is postulated as one explanation for the increased sensitivity to hypoxia in diabetic subjects, leading to tissue dysfunction and injury. Our findings of an increase in the proliferation of fibroblasts from both normal skin and chronic diabetic wounds when exposed to HBO are in line with animal experiments on the healing of both ischemic and nonischemic artificial wounds, which have shown that both kinds of wounds are positively influenced by HBO.

Normal wound healing is regulated by a number of growth factors which stimulate or inhibit the cells to migrate, proliferate, synthesize extracellular matrix components, and exhibit other properties. Our findings indicate that HBO stimulates fibroblast proliferation and thereby this effect could contribute to granulation tissue formation. This could indirectly or directly be explained by activation of mitogenic growth factors by HBO. In addition, the effects could be due to an increased oxygen availability.

It has been reported that cells cultured in ways similar to ours suffer from relative hypoxia. However, the
dose-dependent effect of a single 1-hour exposure to oxygen suggests a pharmacologic effect of oxygen on the cells more than just increased availability of oxygen. So does our finding that high doses of $O_2$ are needed to enhance diabetic chronic wound fibroblast proliferation. Furthermore, in vitro studies on mouse fibroblast cell proliferation and collagen biosynthesis have shown growth to be maximized in cultures maintained at a constant $P_O_2$ of 5 to 10 kPa (37.5 to 75 mm Hg) from 1 to 4 days. At higher oxygen levels (21 to 96 kPa), oxygen toxicity reduced growth rate and cells were killed within 24 hours when maintained at 238 kPa. Hence, we suggest that a short-term HBO exposure may initiate intracellular events that stimulate fibroblast proliferation.

We suggest that the effects of oxygen on fibroblast proliferation may be one of the major contributing factors for the positive influence of HBO treatment on the healing of hypoxic wounds. Further studies are needed to explain the molecular mechanisms responsible for these observations.

ACKNOWLEDGMENTS

We are grateful to the Swedish Medical Research council (grant No. 018406), AGA AB, Swedish Fund for Research Without Animal Experiments and to Mr. Erling Persson for financial support. We also thank Kerstin Plahn and Eva Fagerlund for technical assistance with the monoplace chambers.

REFERENCES