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Effect of Oxygen on Phagocytic and Adherence Functions in Mouse Spleen Macrophages

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Mehm WJ, Pimsler M. Effect of oxygen on phagocytic and adherence functions in mouse spleen macrophages. *J Hyperbaric Med* 1986; 1(4):223–231.—The effects of varied oxygen partial pressures were tested on the phagocytic and adherence functions of mouse spleen macrophages. The evaluation was accomplished in vitro using standard cytologic and microscopic techniques. Macrophages were harvested from mouse spleens, placed in culture on glass coverslips and fed latex beads, and allowed to feed for varying time intervals. During the feeding period, cultures were exposed to one of several oxygen partial pressures via an environment chamber or hyperbaric chamber. After treatment, macrophages were fixed and stained and cell counts accomplished to record the total number of macrophages adhered and the total number of bead-positive macrophages present in a sample of 20 fields/slide. Our data indicate that short-term exposure of mouse spleen macrophages to relatively high oxygen tensions does not affect either their rate of phagocytosis or adherence to glass slides. These findings suggest that it is not the effect of oxygen on macrophage adherence or phagocytic activity during wound debridement that is responsible for enhanced wound healing in response to hyperbaric oxygen therapy.

hyperbaric oxygen therapy, hyperoxia, wound healing, phagocytosis, cell adherence, cell culture.

Introduction

Wound healing is a tightly orchestrated process involving multiple interacting cell types and biochemical mediators. Following tissue injury, platelet-derived factors (1–4), fibrin (5), and the split products of fibrin and complement (6) act as chemoattractants, recruiting macrophages, fibroblasts, smooth muscle cells, and endothelial cells to the wound site. These cells become organized (form the healing module or wound module), interact, and produce cytokines, which stimulate cell growth, recruit other cells, and promote angiogenesis and collagen production (5, 7–9). Macrophages phagocytize dead tissue and contaminants, debriding the wound, and the dead areas become filled with granulation tissue (10). Finally, the wound site is repopulated by cells normally present at the site (epidermal cells in skin and parenchymal cells in internal organs). Thus the original tissue is reconstituted (6).

Although oxygen is known to play a central role in this complex process (11–16), there remains a vital need to define the component parts of wound

healing and to integrate them into a coherent model whereby their mechanisms and interactions are understood. Of particular interest to our group is the effect of oxygen on the role played by the macrophage in wound debridement. Macrophages, via phagocytosis, debride the wound by removing infecting organisms and eliminating necrotic tissue from the site. Removal of the infectious material and of the devitalized tissue ultimately clears the way for tissue reconstruction. A previous study has indicated that the rate of wound debridement is determined by the number of macrophages present in the circulatory system and at the wound site (10). Studies of alveolar macrophages demonstrated reductions in their bactericidal activity as a result of cigarette smoke (17) and an up to 50% impairment of phagocytosis, adherence, chemotaxis, degranulation, and bactericidal activity when the macrophages were exposed to hyperoxia (18).

Our laboratory has begun to investigate the role of macrophages in wound healing by studying the effects of varying oxygen tensions on the phagocytic and adherence functions of mouse spleen macrophages in vitro. This first report focuses on two specific questions. To what extent do the partial pressure of oxygen and time of exposure affect: a) adherence of macrophages to glass slides, and b) the rate of macrophage phagocytosis measured by latex bead ingestion? Better understanding of these areas will help delineate cellular and molecular mechanisms involved in wound healing and eventually aid in the development of rational designs for more effective hyperbaric oxygen therapies.

Materials and Methods

The study consisted of exposing cultures of mouse spleen macrophages to combinations of 5 partial pressures of oxygen: 2280, 1520, 760, 160, and 38 mmHg, at 4 exposure times: 30, 60, 90, and 120 min. During these oxygen exposure periods, macrophages were incubated with latex beads. For each of the 20 combinations of oxygen partial pressures and exposure times, 12 slides were individually replicated and measured with regard to adherence (total number of macrophages adhering to 20 randomly selected 400× microscope fields per slide) and to phagocytosis (percentage of bead-positive macrophages occurring in the 20 selected fields). Bead-positive cells are those that contain five or more latex beads.

Measurement of P_{O_2} *in Culture Medium*

It was necessary to determine that our protocol resulted in significant differences in levels of dissolved O_2 in the culture medium. To this end we incubated 6-well (30mm diameter) Costar plates. Each well was filled to a depth of 2 mm with culture medium under the 5 conditions of differing oxygen overpressures for 60 min (38, 160, 760, 1520, and 2280 mmHg). The plates were then removed from the chamber or from the environment box

and the P_{O_2} measured within 2 min using a polarigraphic O_2 electrode (Watanabe, Inc., Tehachapi, CA). The data in Table 1 represent the means and SD for a minimum of 3 replicate exposures at each oxygen overpressure. These data demonstrate that in 1 h significant levels of dissolved O_2 are attained under these conditions and that there is a general relationship between oxygen overpressure and P_{O_2} in solution. Dissolved P_{O_2} ranged from a low of 50 mm at 38 mmO_2 overpressure to a high of 1245 mm at 2280 mmO_2 overpressure.

Animals and Treatment Exposures

For the study, 200 conventional BALB/c male mice (Ace Animals, Boyertown, PA), 3- to 5-mo.-old were used. Upon receipt, the mice were housed 5 per cage in standard wire cages, fed a balanced food diet of Lab Rodent Chow (Ralston Purina Co., St. Louis, MO), and provided water ad libitum. A 12-h light (6 a.m.–6 p.m.) and 12-h dark cycle was maintained. Room temperature was kept at 22°C and humidity maintained at 50%. In all instances, animals were allowed a 5-d adjustment period before use, which permitted their stress levels to return to normal.

Mice were divided into 4 experimental groups and 1 control group by oxygen partial pressure (40 mice each), as shown in Table 2. These groups were further divided into 4 exposure-time intervals of 30, 60, 90, and 120 min, each containing 10 mice. These exposure times were selected to reflect current hyperbaric oxygen treatment regimens which do not exceed human tolerance

Table 1: Dissolved oxygen levels as a function of oxygen overpressure (mmHg)

	1 ATA, 5%,[a] 38 mm	1 ATA, 21%, 160 mm	1 ATA, 100%, 760 mm	2 ATA, 100%, 1520 mm	3 ATA, 100%, 2280 mm
Mean	50.0	118.3	439.2	699.4	1245.0
SD	12.3	7.0	27.8	52.7	45.0
n	3	3	6	9	6

[a]Percentages refer to the percent of oxygen in the gas mixture.

Table 2: Experimental design—oxygen exposure conditions

P_{O_2}	% Oxygen	Exposure	Condition	ATA[a]
2280	100	hyperbaric	extreme hyperoxia	3
1520	100	hyperbaric	increased hyperoxia	2
760	100	normobaric	hyperoxia	1
160	21	normobaric	atmospheric conditions	1[b]
38	5	normobaric	normal tissue	1

[a]Atmosphere absolute. [b]Experimental control.

limits. Cultures of mouse spleen macrophages were then prepared for these 20 treatment groups, incubated with latex beads, and placed into one of three exposure environments: standard incubator (atmospheric conditions, control), hyperbaric chamber (extreme and increased hyperoxia), or an environment box (hyperoxia and normal tissue). All hyperbaric exposures and the hyperoxia exposure under normobaric conditions consisted of 100% oxygen; 21% O_2 was achieved under normobaric conditions using room air; and the 5% O_2 exposure contained 95% N_2.

Culture Techniques

For each exposure treatment, 10 mice were killed by cervical dislocation and their spleens removed in accordance with procedures employed by our laboratory (19). Cells were teased from spleens in cold Hanks' balanced salt solution, HBSS (Advanced Biotechnologies, Silver Spring, MD), and the suspension thoroughly agitated with a pipette and placed on ice for 15 min. The adherent cells were then allowed to settle. The supernatant was decanted and the remaining cells incubated in 10 ml of medium (RPMI 1640) (Advanced Biotechnologies, Silver Spring, MD) with 5 mg collagenase (Sigma, St. Louis, MO) and 1 mg DNAse (Sigma) for 15 min at 37°C. Cells were washed twice in HBSS and centrifuged at 1500 rpm for 10 min. Adherent cells were then suspended in complete medium [RPMI supplemented with 10% fetal bovine serum (FBS), Flow-Laboratories, McLean, VA] to a concentration of 10^7 cells/ml using a hemacytometer. Two milliliter of the cell suspension was placed on glass coverslips (22 × 22 mm) contained in each of 12 wells (30 mm diameter) in two 6-well Costar plates. Plates were incubated for 2 h at 37°C for adherence selection.

Following incubation, coverslips were washed 3 times in HBSS and agitated with a pipette to remove all nonadherent cells. Two milliliter of complete medium containing a 0.2% concentration of latex beads (2.84 μm diameter, Dow Chemical for Seragen Diagnostics, Indianapolis, IN) was added to each Costar well with coverslip. Plates were incubated for 30, 60, 90, and 120 min at various oxygen tensions at 37°C in the exposure environments outlined above. This procedure allowed macrophages in the 20 respective treatment groups to phagocytize beads as a function of time and oxygen tension. After exposure, coverslips were washed vigorously in HBSS to remove nonphagocytized beads, fixed in acetone for 30 s, stained for nonspecific esterase (20), and mounted on slides. To ensure that beads observed in macrophages were in fact phagocytized by macrophages and not simply adhered to the cell surface, parallel cultures were maintained at 4°C to eliminate active phagocytosis. No intracellular latex beads were observed in this group.

Data Acquisition

Cell counts. The total macrophages (esterase-positive cells) present in 20 randomly selected microscopic fields were counted per slide and recorded

as bead positive or bead negative. Cell counts were obtained by sampling 20 microscopic fields at 400×, five fields from each of four rows. The first field was located in the upper-left corner of the coverslip, four fields down from the top and four fields to the right of the left coverslip margin. The second row sampled was located four fields down from the previous row. Measurements of macrophage adherence were expressed as average number of cells per 20 microscopic fields ± SD. Measurements of phagocytosis were expressed as percentage of bead-positive cells per 20 microscopic fields ± SD.

Statistical analysis. To determine the effects of oxygen tension and exposure time on each of the two response variables (total cell adherence and percent bead-positive cells), a two-factor analysis of variance (ANOVA) was performed. A square root transformation of the cell adherence data was necessary to achieve a variable with homogeneous variance. No transformations were performed on the percent bead-positive cell data. Statistical significance was set at the 95% confidence level.

Results

In all experiments, coverslip-adherent cells were pulsed with uniform diameter latex beads for 30, 60, 90, or 120 min under varying partial pressures of oxygen. The coverslips were then fixed in acetone, stained for nonspecific esterase, and examined microscopically. Macrophages (esterase-positive cells) were scored as either bead positive (5 or more beads ingested) or bead negative (fewer than 5 beads ingested). Effects of each time-oxygen combination were determined in 12 replicate cultures.

Cell Adherence

The total macrophages in 20, 400× fields were counted on each of 12 coverslips representing each exposure-oxygen combination. The results of this study are expressed as the mean and SD of the 12 replicates and are presented in Fig. 1. A great deal of variability occurred in the total number of macrophages counted on coverslips in each group. This is evident from the large standard deviations, many of which exceed 50% of the mean. ANOVA identified a few instances where varying conditions produced statistically significant differences between groups (for example, between 38 vs. 2280 mm at 30 min or between 30, 60, and 120 min at 760 mm), but covariate analysis revealed no significant relationship between time of exposure and oxygen partial pressure. The observed differences follow no consistent pattern and probably reflect statistical "noise" and experimental variability. There does not seem to be a biologically significant effect of time of exposure to varied oxygen tensions on the adherence of murine stromal macrophages in this system.

The overall trend is for the number of macrophages counted to decrease with increasing exposure time. This is probably because after 120 min some

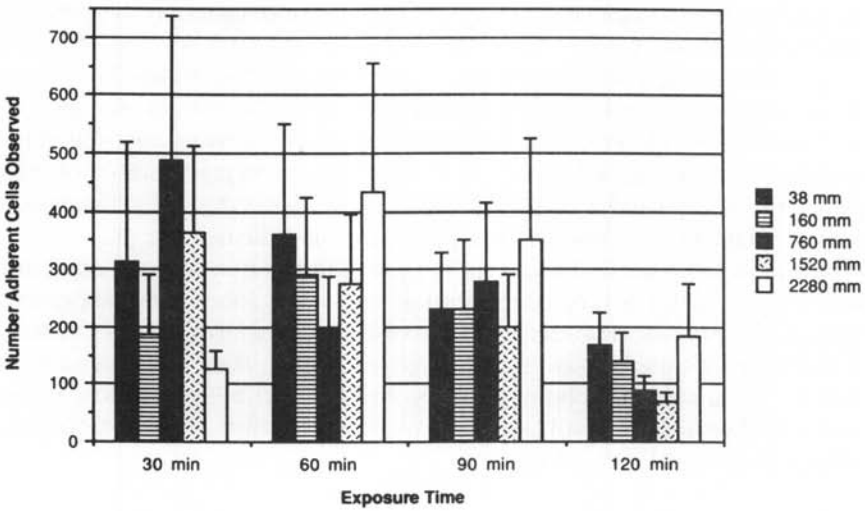

FIG. 1—Mean number of adherent macrophages ± SD per slide (n = 12) for 5 partial pressures of oxygen (mmHg) at 4 exposure times.

macrophages have ingested so many latex beads that they can no longer remain attached to the coverslip. This trend, however, was not statistically significant.

Phagocytosis

Figure 2 illustrates the mean percentage of bead-positive macrophages present as a function of oxygen partial pressure and exposure time. These cells ranged from 12.3% (38 mm) to 32.9% (160 mm) at 30 min, from 45.3% (160 mm) to 75.4% (760 mm) at 60 min, from 74.0% (1520 mm) to 85.3% (2280 mm) at 90 min, and from 58.4% (38 mm) to 68.2% (1520 mm) at 120 min. No consistent pattern was observed, and ANOVA revealed no significant differences among the 60-, 90-, or 120-min exposure groups. Bead-positive macrophages were significantly fewer at 30 min for all groups than in groups with longer exposure times ($P < 0.05$), which suggests that 30 min is insufficient time for most macrophages to ingest 5 or more latex beads under any oxygen condition. Highest values were observed at 60 and 90 min, with most groups demonstrating maximum bead ingestion at 90 min.

Discussion

Hyperbaric oxygen therapy has been shown to be an effective modality for treatment of nonhealing wounds in which microcirculation has been impaired. Hyperoxia apparently increases tissue oxygen levels at the wound site, promoting respiration and enhancing oxygen-requiring chemical reactions (e.g., synthesis of hydroxyproline, etc.). However, the specific actions of elevated

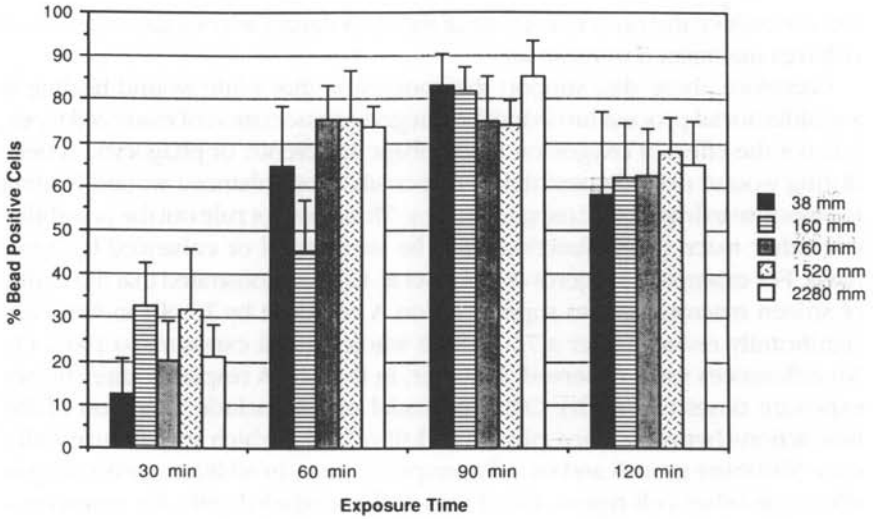

FIG. 2—Mean percentage of bead-positive macrophages ± SD per slide (n = 12) for 5 partial pressures of oxygen (mmHg) at 4 exposure times.

oxygen tension on the cells participating in wound healing are not well defined. Our investigation on the in vitro effects of hyperoxia on macrophage function suggests that short-term exposure to relatively high oxygen tensions does not affect either phagocytosis of latex beads or glass adherence by mature stromal macrophages from mouse spleen.

These results differ from those of Rister (18), who reported that prolonged (up to 96 h) in vitro exposure of guinea pigs to 85% O_2 resulted in decreased phagocytosis and adherence by alveolar macrophages in vitro. However, there are several significant differences between our methods and those of Rister. First, Rister used alveolar macrophages exposed for prolonged periods of time in vivo to elevated oxygen tensions rather than tissue macrophages exposed for short periods in vitro. Alveolar macrophages are in direct contact with air on the alveolar surface and are thus more exposed and susceptible to oxygen damage than are tissue macrophages. Second, Rister measured phagocytosis of oil red O paraffin droplets spectrophotometrically in cell suspensions. We have observed that macrophages are far more active phagocytically when adhered to glass or plastic surfaces than when in suspension (Pimsler, personal observation). Finally, Rister's assessment of adherence was based on short-term (5 min) association of macrophages with nylon wool, and did not examine the effects of hyperoxia on already adherent macrophage cultures. Our observation that adherence is not adversely affected by hyperoxia is supported by Bowles et al. (21), who reported that in vitro exposure of glass-adherent alveolar macrophage cultures to 100% oxygen for up to 3 h

did not reduce the protein content of the monolayers as compared to control cultures maintained in room air.

Therefore, these data support the conclusion that while wound healing is a multifactorial process involving the integrated association of many cell types, it is not the effect of oxygen on macrophage adherence or phagocytic activity during wound debridement that is responsible for enhanced wound healing in response to hyperbaric oxygen therapy. This does not rule out the possibility that other macrophage functions may be suppressed or enhanced by hyperoxia. For example, Gougerot-Pocidalo et al. (22) demonstrated that the ability of spleen macrophages to support a Con A response by T cells in vitro was significantly reduced after a 72- to 96-h whole-animal exposure to 100% O_2. No differences were observed, however, in the Con A response after shorter exposure times (24–45 h). Other potential effects include alteration of the interactions between macrophages and fibroblasts, which may in turn influence fibroblast growth and/or collagen production. In addition, direct oxygen effects on other cell types—fibroblasts and endothelial cells, for example—cannot be discounted. Any changes in the relationships among these cells may alter the healing process via fibroblast growth and/or collagen production, rate of wound closure, cell proliferation, or angiogenesis. Therefore, in vitro studies of these and other tissues are needed to better understand the complex cell interactions involved in wound healing, and to determine how these cellular functions may be promoted by hyperbaric oxygen therapy.

References

1. Deuel TF, Senior RM, Chang D, Griffin G, Hemmilsson RL, Kaiser ET. Platelet factor 4 is chemotactic for neutrophils and monocytes. Proc Nat Acad Sci USA 1981; 78:4584–5601.
2. Grotendorst GR, Seppa HEJ, Kleinman HK, Martin GR. Attachment of smooth muscle cells to collagens and their migration toward platelet derived growth factors. Proc Nat Acad Sci USA 1981; 78:3669–3672.
3. Grotendorst GR, Chang T, Seppa HEJ, Kleinman HK, Martin GR. Platelet-derived growth factor is a chemoattractant for vascular smooth muscle cells. J Cell Physiol 1982; 113:261–266.
4. Seppa HEJ, Grotendorst GR, Seppa SI, Sheiffmann E, Martin GR. The platelet-derived growth factor is a chemoattractant for fibroblasts. J Cell Biol 1982; 92:584–588.
5. Hunt TK. Can repair processes be stimulated by modulators (cell growth factors, angiogenic factors, etc.) without adversely affecting normal processes? J Trauma 1984; 24(Suppl 9):-S39–S49.
6. Hunt TK, Knighton DR, Thakral KK, Andrews WS, Michaeli D. Cellular control of repair. In: Hunt TK, Heppenstall RB, Pines E, Rovee D, eds. Soft and hard tissue repair, surgical sciences series, vol. 2. New York: Praeger Publishers: 1984:3–19.
7. Wahl SM. Role of mononuclear cells in the wound repair process. In: Dineen P, Hildick-Smith G, eds. The surgical wound. Philadelphia: Lea and Febiger; 1981:63–74.
8. Grotendorst GR, Pencev D, Martin GR, Sodek J. Molecular mediators of tissue repair. In: Hunt HK, Heppenstall RB, Pines E, Rovee D, eds. Soft and hard tissue repair, surgical sciences series, vol. 2. New York: Praeger Publishers; 1984:21–48.
9. Hunt TK, Andrews WS, Halliday B, et al. Coagulation and macrophage stimulation of angiogenesis and wound healing. In: Dineen P, Hildick-Smith G, eds. The surgical wound. Philadelphia: Lea and Febiger, 1981.

10. Leibovich SJ, Ross R. The role of the macrophage in wound repair: a study with hydrocortisone and antimacrophage serum. Am J Pathol 1975; 78:71–100.
11. Hunt TK, Conolly WB, Aronson SB, Goldstein P. Anaerobic metabolism and wound healing: A hypothesis for the initiation and cessation of collagen synthesis in wounds. Am J Surg 1978; 135:328–332.
12. Hunt TK, Linsey M, Grislis G, Sonne M, Jawetz E. The effect of differing ambient oxygen tensions on wound infection. Ann Surg 1975; 181:35–39.
13. Hunt TK, Pai MP. The effect of varying ambient oxygen tensions on wound metabolism and collagen synthesis. Surg Gynecol Obstet 1972; 135:561–567.
14. Hunt TK, Zederfeldt B, Goldstick TK. Oxygen and healing. Am J Surg 1969; 118:521–525.
15. Kivisaari J, Niinkoski J. Effects of hyperbaric oxygenation and prolonged hypoxia on the healing of open wounds. Acta Chir Scand 1975; 141:14–19.
16. Kivisaari J, Vihersaari T, Renvall S, Niinkoski J. Energy metabolism of experimental wounds at various oxygen environments. Ann Surg 1975; 181:823–828.
17. Thomas WR, Holt PG, Keast D. Cigarette smoke and phagocyte function: Effect of chronic exposure *in vivo* and acute exposure *in vitro*. Infect Immun 1978; 20:468–475.
18. Rister M. Effects of hyperoxia on phagocytosis. Blut 1982; 45:157–166.
19. Pimsler M, Trial J, Forman J. Antigen-presenting cells that induce anti-H-2K T-cell responses: Differences in stimulator-cell requirements for induction of proliferation and cell-mediated lympholysis. Immunogenetics 1981; 12:297–312.
20. Yam LT, Li CY, Crosby WH. Cytochemical identification of monocytes and granulocytes. Am J Clin Pathol 1971; 55:283–290.
21. Bowles AL, Dauber JH, Daniele RP. The effect of hyperoxia on migration of alveolar macrophages *in vitro*. Am Rev Respir Dis 1979; 120:541–545.
22. Gougerot-Pocidalo MA, Fay M, Pocidalo JJ. *In vivo* normobaric oxygen exposure depresses spleen cell *in vitro* Con A response. Effects of 2-mercaptoethanol and peritoneal cells. Clin Exp Immunol 1984; 58:428–435.

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