OXYGEN MODULATES GROWTH OF HUMAN CELLS AT PHYSIOLOGIC PARTIAL PRESSURES

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Oxidative stress derives from two sources: (*a*) ambient oxygen present in concentrations greater than that required for respiration; and (*b*) oxidative intermediates produced as a consequence of respiration. The oxygen tension available to differentiating tissue influences the process of differentiation and can affect the final cell type produced (1, 2). Reactive oxygen radicals formed from reductive intermediates of oxygen during normal cellular metabolism damage cells and could contribute to the aging process (3–5). Biochemical reactions comprising oxidative phosphorylation produce and release a substantial number of reactive free radical intermediates (7).

We are testing the hypothesis that oxidative stress contributes to the aging process. We have developed a cell culture system to define the consequences of the interaction between oxygen and cellular metabolic processes and ascertain the response of human cells to the additional oxidative stress imposed by increased partial pressures of oxygen. Earlier we found that elevated oxygen tensions are progressively toxic to human cells in culture; we have begun to explore the mechanisms of this toxicity and the ability of the cell to protect itself from the oxidative stress constantly present in the environment (7–9). Several investigators (10–12) have recently observed increased clonal growth of cells when they are cultivated at partial pressures of oxygen below that of the ambient atmosphere. The growth-promoting effects of reduced oxygen tensions on clonal growth were greater than those we have observed in cells seeded at high density in mass culture. We have therefore investigated the influence of seeding density on the growth of embryonic human fibroblasts at different partial pressures of oxygen (Po_2).[1] We have found that oxygen regulates the growth of human cells under physiologic partial pressures and that oxygen toxicity contributes to the seeding density dependence of cellular growth commonly seen in cell culture.

Materials and Methods

The human diploid embryonic lung fibroblast cell strains WI-38 and IMR90 were obtained from Dr. Vincent Cristofalo of the Wistar Institute, Philadelphia. Cells were grown in Dulbecco's modified Eagle's medium (DME) (1 gm/liter glucose; Gibco Laboratories, Grand Island, NY) (13). Immediately before use, the medium was supplemented

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[1] *Abbreviations used in this paper:* DME, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; Pco_2, partial pressure of carbon dioxide; PH_2O, partial pressure of water vapor; Po_2, partial pressure of oxygen.

with L-glutamine (2 mm) (Flow Laboratories, Rockville, MD) and fetal bovine serum (FBS) (10% vol/vol) (Flow Laboratories). Antibiotics were not normally used.

Cultures were grown at 37°C in 75-cm^2 sealed polystyrene flasks (No. 3024; Falcon Labware, Oxnard, CA) containing 40 ml medium (0.54 ml/cm^2) (14) and with a gas phase volume of 233 cc. In some experiments cells were grown in 25-cm^2 polystyrene flasks (Lux No. 5350; Miles Laboratories, Naperville, IL) containing 13 ml (0.54 ml/cm^2) and with a gas phase volume of 49 mm. In others we used hydrophilic Petriperm dishes (Tekmar Co., Cincinnati, OH), petri dishes with a 25 μm thick Teflon membrane as the cell support surface. This membrane is freely permeable to oxygen; the dishes had 20 cm^2 of growth area and contained 10 ml of medium. The dishes were placed on perforated stainless steel trays to allow free diffusion of gas to the entire growth surface. Petriperm dishes were placed in the incubators immediately after seeding to minimize exposure to room air (which does not contain 5% carbon dioxide). Antibiotics (penicillin, streptomycin, fungizone) were used in some experiments performed in Petriperm dishes. In all three types of vessels, a 4.9–5.1-mm layer of medium covered the cell sheet.

Routinely confluent monolayers were subcultivated weekly. The cells were released from the plastic with trypsin (0.25%) (Flow Laboratories) in Ca^{++}- and Mg^{++}-free Hanks' balanced salt solution (M. A. Bioproducts, Walkersville, MD). After suspension in medium containing 10% FBS, the cells were counted and inoculated into appropriate vessels at a density of 10^4 cells/cm^2. A Coulter Counter (Coulter Electronics, Hialeah, FL) was used for cell counts. Cells were normally grown without shaking in a sealed atmosphere of 5% CO$_2$, 20% O$_2$, and 75% N$_2$. Cultures not confluent after 7 d were fed every 7 d until either confluence or phase-out. Phase-out was defined as the inability to achieve confluence after four feedings over a 4-wk period.

Population doublings were calculated in the standard way by comparing cell counts per vessel at seeding with counts at confluence (15). In some experiments, described below, population doublings were determined by comparing cell counts of attached surviving cells 18 h after subcultivation with counts at confluence. We determined the percentage of labeled nuclei autoradiographically by the method of Cristofalo and Sharf (15) and monitored for mycoplasma contamination by the method of Schneider and Stanbridge (16).

Routine Gassing Procedure. Both before and after seeding, flasks were gassed to equilibrium with analyzed and certified standard gas mixtures (Matheson Gas Products, East Rutherford, NJ). Each mixture contained 5% CO$_2$, a different percentage of oxygen (0, 1, 5, 10, 15, 20, 30, 40, 50, 75, or 95%), and nitrogen to balance. After seeding and gassing, cells were kept stationary in 1 of 10 incubators (Heraeus; Tekmar Co.) maintained electronically at the oxygen tension used to equilibrate the flask. Each incubator was equipped with a Clark oxygen electrode and a CO$_2$ thermal conductivity detector to maintain the desired percentages of atmospheric oxygen and carbon dioxide. Incubators were calibrated each day by withdrawing a sample of gas and measuring the partial pressure of oxygen and carbon dioxide with a blood gas analyzer (model 113; Instrumentation Laboratory, Inc, Lexington, MA).

Effect of Oxygen on Cell Growth. Actively proliferating cells, seeded at different densities into tissue culture flasks, were equilibrated with an atmosphere of 5% carbon dioxide and different percentages of oxygen. To ensure uniformity of the desired seeding density throughout each experiment, after every 10 flasks seeded, an aliquot of cells was pipetted directly into counting fluid (Isoton; Tekmar Co.) for electronic counting. The standard deviation of the actual seeding density was <10%.

Oxygen tension of the medium in each flask at harvest was measured as described previously (7) with the blood gas analyzer. At an atmospheric pressure of 760 mm Hg, a gas mixture containing 5% CO$_2$, 20% O$_2$, and 75% N$_2$, equilibrated in a flask with medium at 37°C, would give a partial pressure of water vapor (P$_{H_2O}$) of 47, a partial pressure of carbon dioxide (P$_{CO_2}$) of 35.6 mm Hg, a P$_{O_2}$ of 142.6 mm Hg, and a partial pressure of nitrogen of 534.8 mm Hg.

Effect of Oxygen on Cell Culture Medium. We tested the effect of oxygen on the medium,

using flasks preincubated at 37°C for 7 d with complete medium and serum but without cells at either 0, 20, or 95% O_2 (groups 1, 2, and 3) in incubators appropriate to their oxygen tension. Another group of flasks was incubated at 95% O_2 and seeded with cells. No growth occurred at this oxygen tension (Fig. 1), as measured by a direct cell count on day 7. The medium from these flasks was used for further experiments as a fourth category (group 4) of preincubated medium. On day 7, medium from the flask in which growth had been inhibited by 95% O_2 was poured into new T-75 Falcon flasks. On day 7, all flasks received fresh glutamine and the atmosphere was equilibrated with 20% O_2. At that time an additional set of flasks (group 5) was prepared with fresh medium and equilibrated with 20% O_2. Cells were seeded at 10^4 cells/cm^2 into 15 flasks in each of the five groups and their growth was measured over the subsequent 250 h.

Effect of Oxygen on Cell Growth in Shaken and Stationary Vessels. We compared cell growth in flasks that were stationary with that in flasks that were shaken. Shaken flasks were incubated in an environmentally controlled room (37°C) on a rocker platform (Bellco Glass, Vineland, NJ) adjusted to flood cells with medium 10 times per minute over a 60° arc. The stationary cultures were incubated in the same room.

Results

The data from all experiments were reproducible, having been confirmed at least three times by three individuals. Fig. 1 *A–E* depicts the results of a representative series of experiments showing the influence of oxygen on the growth of actively proliferating human diploid fibroblasts at different seeding densities.

Fig. 1 *A* illustrates the effect of oxygen on the growth of WI-38 cells inoculated at 10^4 cells/cm^2. This seeding density is commonly used by others in studies of growth proliferation and cellular aging and was used by us in our own previously reported studies.

As we and others have found, ~50–70% of the inoculated cells attached during the first 18 h after subcultivation. In multiple experiments we found little consistent difference in cell attachment at the different oxygen tensions at any of the seeding densities used. At lower oxygen tensions, exponential growth began ~24 h after inoculation and continued for 3–4 d, yielding ~10^5 cells/cm^2 (Fig. 1*A*). At a seeding density of 10^4, cell growth was similar and maximal between Po_2 14 and 96 mm Hg.

Growth was progressively inhibited at higher oxygen tensions. The population doubling time of cells at higher Po_2 decreased from 28 h at Po_2 140 to 37 h at Po_2 200, 60 h at Po_2 252, and 148 h at Po_2 330 mm Hg. There was no growth at Po_2 577 mm Hg.

As the seeding density was progressively decreased, growth was inhibited by lower Po_2 (Fig. 1*B–E*). Fig. 1*E* illustrates the effect of oxygen on cell growth at 50 cells/cm^2. Maximal growth was achieved at Po_2 9–16 mm Hg, with cell density reaching 2 × 10^4 by 300 h, an increase of ~8.6 population doublings. Inhibition of cellular growth was clear in the cultures grown at a Po_2 of 42, the cells achieving ~2.5 × 10^3 cells/cm^2 at 300 h, ~5.7 population doublings. At Po_2 78 and 104 mm Hg, cells grew similarly and were quite depressed compared with growth at the lower oxygen tensions, achieving a density of 7 × 10^2 by 300 h, ~3.8 population doublings. Cells grown at Po_2 138 mm Hg achieved a density of only 4 × 10^2 cells/cm^2 by 300 h, ~3 population doublings. Here again, as the oxygen tension was further increased, growth was retarded. Therefore, we see clearly in Fig. 1 *A–E* that as the seeding density was decreased, cellular growth was inhibited by progressively lower partial pressures of oxygen.
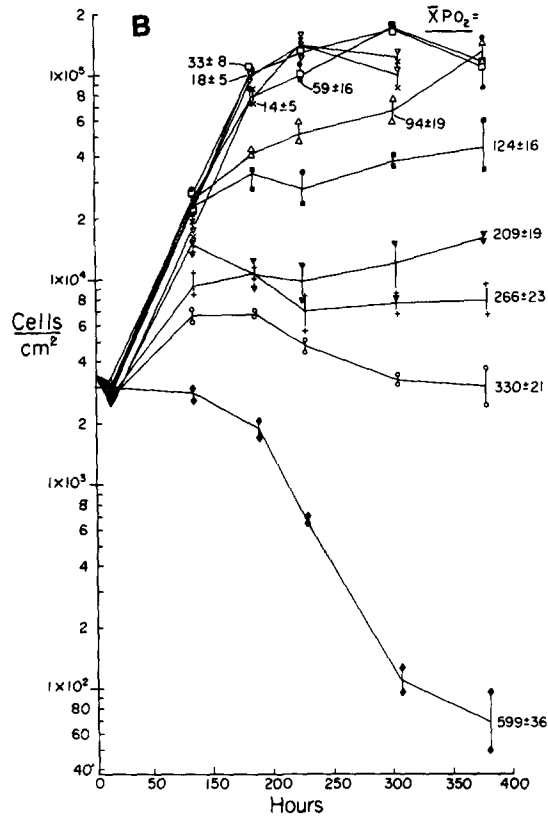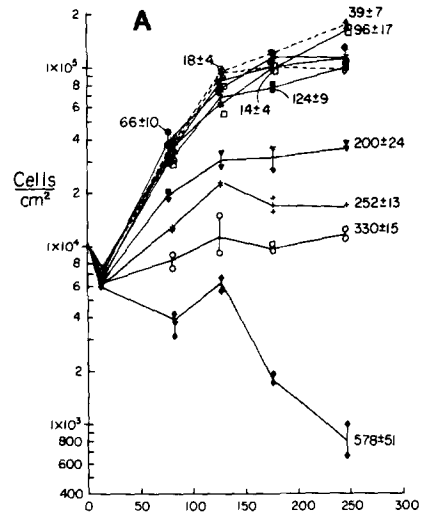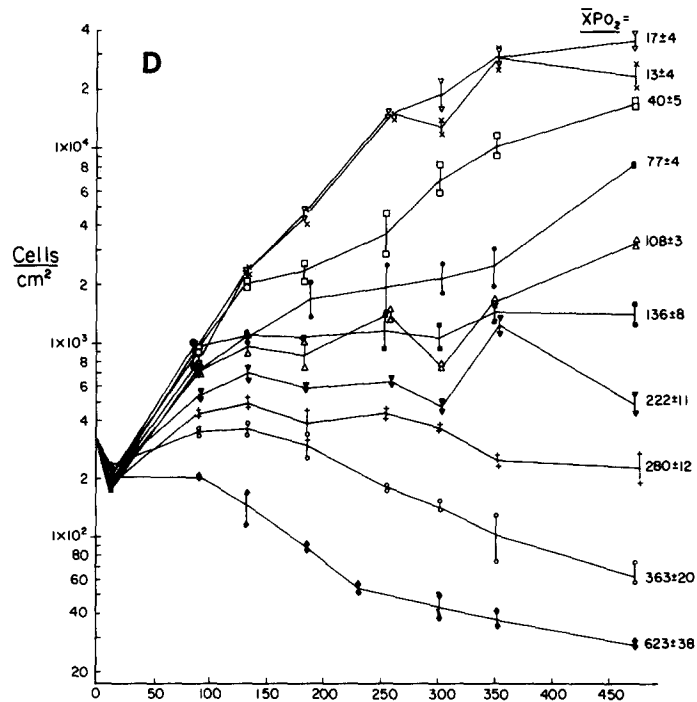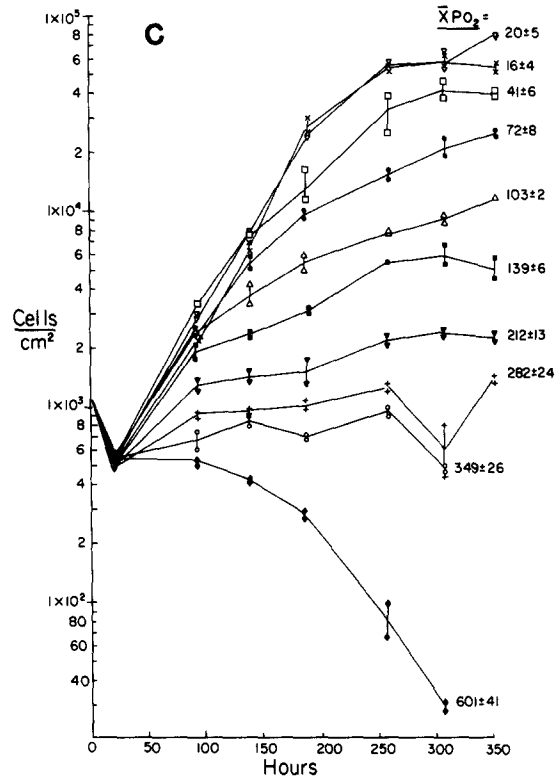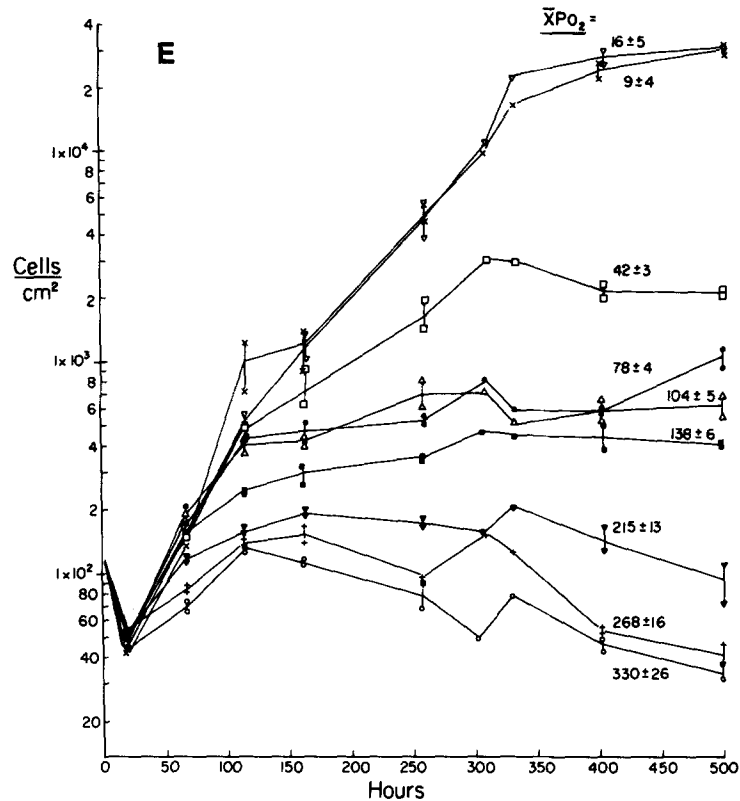
FIGURE 1. (A-E) Effect of oxygen tension on the growth of human diploid fibroblasts inoculated at different cell densities. Mid-passage WI-38 cells (75-90% labeled nuclei) were seeded into polystyrene tissue culture flasks, equilibrated with atmospheres containing 5% carbon dioxide and different partial pressures of oxygen; and incubated at 37°C. Flasks contained 0.53 ml/cm^2 of media (DME + 10% fetal calf serum). Before each flask was harvested, the actual partial pressure in the media of that flask was determined using a blood gas analyzer. Numbers associated with each line represent average partial pressure of oxygen in millimeters of Hg of media of each flask used to construct the line. Vertical lines connect replicate points at each oxygen tension and time interval and indicate the spread of the data obtained. Cells inoculated at (A) 10^4 cells/cm^2, (B) 3.3 × 10^3, (C) 10^3, (D) 3.3 × 10^2, (E) 10^2.

Figs. 2 and 3 show that there is a seeding density influence on the inhibition of cell growth by oxygen at physiologic partial pressures. These data were derived from a single experiment similar to that portrayed in Fig. 1, but differing in that all growth curves were performed simultaneously and were inoculated from the same cell suspension. The data are presented as a ratio obtained by determining the population doubling increase at 7 d at the experimental oxygen tension divided by the population doubling increase at 7 d under 20% O$_2$ (Po$_2$ 132 mm Hg).

Expressed in this manner, the data indicate the amount of growth at each oxygen tension and at each seeding density relative to that obtained under the control Po$_2$ of 130 mm Hg. The values of cell number at 18 h and at 7 d were the means of three flasks for each determination. The results of such an experiment are summarized in Fig. 2 and show that 20% O$_2$ did not inhibit cell
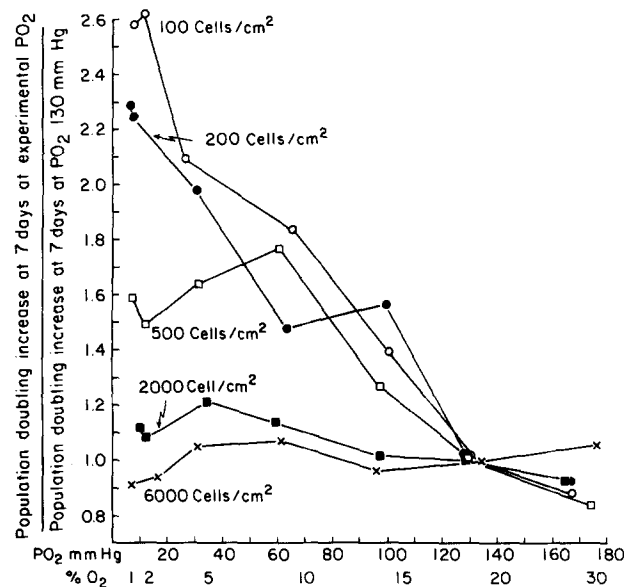
FIGURE 2. Effect of seeding density on the population doubling increase (PDI) at experimental oxygen tensions compared with that at 20% oxygen. PDI at 7 d at the experimental oxygen tension was determined from the mean of three cultures at each time by the following formula: PDI = [ln (cell number at 7 d/cells attached at 18 h)]/ln 2. Population doubling increase at the experimental oxygen tension was divided by population doubling increase at 7 d at 20% oxygen (Po_2 130 mm Hg).

growth when cells are seeded at 6,000 cells/cm^2, but that as the seeding density was reduced cell growth was inhibited by progressively lower oxygen tensions. This figure also illustrates that at seeding densities ranging from 500 to 6,000 cells/cm^2, cell growth was improved over a Po_2 range of 30–60 mm Hg compared with <20 mm Hg. We have previously shown marked inhibition of growth in cultures at 6,000–8,000 cells/cm^2 incubated at Po_2 3–6 mm Hg (7). In other experiments (not shown), densities of 50 and 12,000 cells/cm^2 fall where they would be expected in this progression of curves.

Fig. 3 portrays data derived from Fig. 1 A–E illustrating the effect of seeding density on the growth of cells at 20–95% O_2. At Po_2 >350 mm Hg the inhibition of growth at experimental compared with the control conditions was independent of the seeding density. Growth was similarly inhibited at seeding densities of 12,000 cells/cm^2. Growth inhibition by elevated partial pressures of oxygen is partially reversible. We previously showed that, after 7 d of cultivation at Po_2 560–640 mm Hg, 75–90% of cells retained viability and the cell population could grow again when reequilibrated with lower oxygen tensions (data not shown; see references 7, 8).

Effects of Oxygen on Cell Culture Medium. Inhibition of cellular growth by oxygen is caused by an effect of oxygen on the cells, not on the medium (Fig. 4). Growth was similar for cells incubated in fresh medium and cells incubated in medium that had failed to support cell growth the previous week when incubated under 95% O_2. Furthermore, cell growth in medium preincubated at 95% O_2 without cells was always equal to or better than growth in medium preincubated
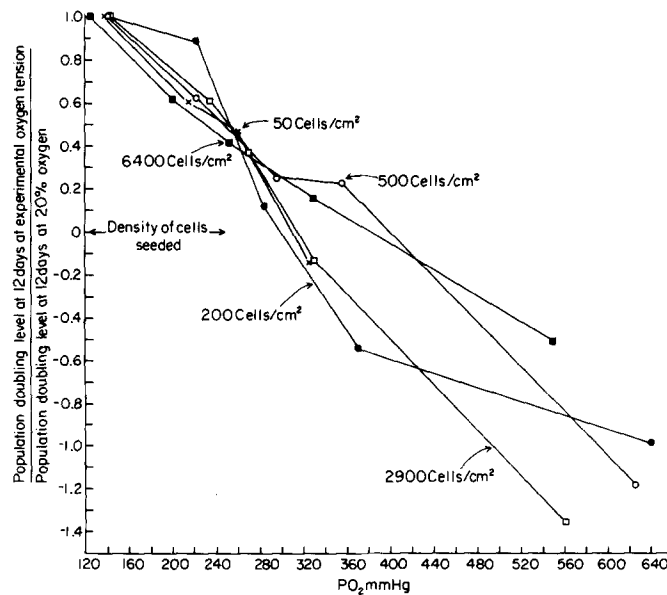
FIGURE 3. Effect of seeding density on population doubling increase (PDI) at experimental oxygen tensions compared with PDI at 20% oxygen. PDI at 12 d at the experimental oxygen tension was determined from the mean of three cultures at each time by the formula: PDI = [ln (cell number at 12 d/cells attached at 18 h)]/ln 2. Population doubling increase at the experimental oxygen tension was divided by population doubling increase at 12 d at 20% oxygen (Po_2 130 mm Hg).

at 20 or 0% O_2. It is thus clear that oxygen does not inhibit cell growth by destroying an essential medium component. Growth in medium preincubated with cells at 95% O_2 was usually better than growth in medium preincubated under any of the oxygen tensions without cells. Growth in medium preincubated at 37°C for 1 wk without cells was usually slightly less than that in freshly prepared medium.

Decreased Oxygen Toxicity at Higher Seeding Densities Is Not Explained by Oxygen Consumption in the Cellular Microenvironment. We asked whether rapid cell growth at 20% O_2 at high seeding densities is caused by reduction in O_2 in the cellular microenvironment by cellular O_2 utilization. At 10^4 cells/cm^2, cells might grow at their maximal rate if they reduce the oxygen tension of the microenvironment. Cells inoculated at lower densities (e.g., 10^2 cells/cm^2) would use insufficient oxygen to establish such diffusion gradients and would not lower the oxygen in their cellular microenvironment. This hypothesis was explored in two ways.

First, we compared the growth in cultures inoculated with 1 or 2 × 10^4 cells/cm^2 and grown at a Po_2 of 140 mm Hg in a stationary set-up or on a rocking platform. The latter caused medium to wash over the cell surface 10 times in 1 min. We reasoned that if oxygen consumption were reducing the effective oxygen concentration in the cellular microenvironment and thereby promoting cell growth, then cells grown stationary at a Po_2 of 140 should grow better than cells incubated on the rocking platform. The rocking platform would prevent diffusion gradients being established in the medium. In addition, differential growth
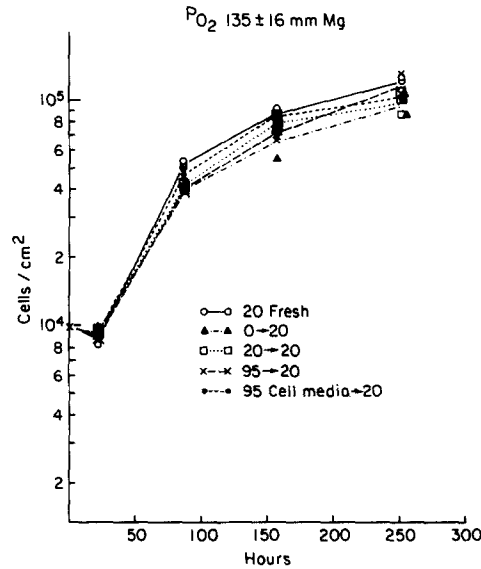
FIGURE 4. Effect of preincubation of media at differennt oxygen tensions at 37°C for 1 wk on subsequent growth of cells. Complete media, including serum, was prepared in T75 flasks, equilibrated with 0% O_2 (▲), 20% O_2 (□); 95% O_2 (×) and incubated at 37°C for 1 wk without cells. An additional set of flasks was equilibrated at 95% O_2, inoculated with cells at 10^4 cells/cm^2, and incubated at 37°C for 1 wk (●). No cell growth occurred in these flasks, as measured by direct cell count on day 7. After day 7, the media was poured off the flasks marked (●) to new flasks, and all flasks were supplemented with fresh glutamine (2 mM). An additional set of flasks was prepared with fresh media (○). All flasks were equilibrated with 20% O_2 (PO_2 135 mm Hg) and inoculated with cells at 10^4 cells/cm^2. Growth was determined over the subsequent 250 h. The curve is drawn through the mean of three replicate flasks for each point.

effects between shaken and stationary cultures should be magnified in cultures inoculated at different densities, because more oxygen would be used by more crowded cultures. Shaking did not affect the cell attachment rate (24-h point, Fig. 5), rate of growth, or final saturation density of cultures incubated at 1 or 2 × 10^4 cells/cm^2. The improved growth seen at higher oxygen tensions with higher seeding densities (Fig. 1 A–E) was thus not caused by a reduction in oxygen tension in the cellular microenvironment.

A second approach was used to confirm this conclusion. Cells were grown on 25-μm Teflon membranes (Petriperm dishes) that are freely permeable to oxygen. Growth on this support surface in conjunction with the shelf design of the incubators exposes cells to the partial pressure of oxygen in the ambient air of the incubator without diffusion gradients. This was confirmed by withdrawing samples of medium from the dishes and comparing the PO_2 and PCO_2 of the medium with that measured in the gas phase of the incubator. Fig. 6 illustrates the growth of young passage IMR90 cells (91% labeled nuclei) seeded at 10^4 (A), 10^3 (B), or 10^2 cells/cm^2 (C) in gas-permeable dishes, as described in Materials and Methods. This experiment confirms the conclusions reached from the shaking vs. stationary experiment. It shows that growth of cells grown on gas-permeable membranes, seeded at 10^4 cells/cm^2 is similar between PO_2 9 and 106
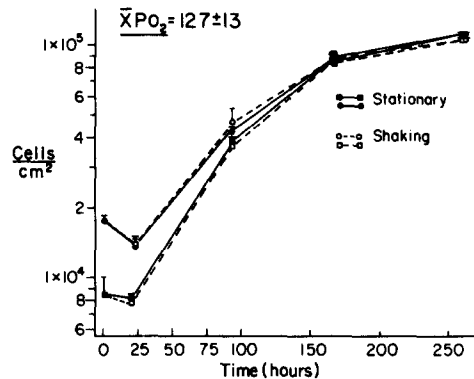
FIGURE 5.   Shaken vs. stationary growth of human diploid fibroblasts inoculated at 10^4 or 2
× 10^4 cells/cm^2 at P_{O_2} 127 mm Hg. Shaken cells were tilted through a 60° arc every 5 s. Each
point represents the mean ± SD of three flasks. The error bars indicate the standard deviation.
Three flasks at each seeding density were shaken for the first 24 h and harvested at 24 h. The
remainder of the shaken flasks were incubated stationary for 24 h and placed on the rocking
platform at 24 h. (□, ■) Inoculated at 1 × 10^4 cells/cm^2; (○, ●) inoculated at 2 × 10^4 cells/
cm^2.

mm Hg and slightly reduced at 143 mm Hg (Fig. 6A). At 10^3 (Fig. 6B) or 10^2
cells/cm^2 (Fig. 6C) the inhibitory effects of oxygen become more prominent at
lower partial pressures of oxygen, as we saw in Fig. 1. We conclude that the
reduced growth inhibition seen at higher oxygen tensions with higher seeding
densities is not due to depletion of oxygen in the cellular microenvironment by
oxygen consumption.

Discussion

We have shown that oxygen modulates the growth of human cells under
physiologic partial pressures, and also that the seeding density dependence of
cellular growth commonly seen in cell culture is partly explained by oxygen
effects. Oxidative free radical reactions constitute a continual peroxidative stress
to living systems and may contribute to the aging process. The intracellular levels
of oxygen needed for respiration are low relative to the concentrations of oxygen
present in the ambient atmosphere. A rat, for example, will continue breathing
until the intracellular oxygen level falls below 0.2 mm Hg (17). Oxygen in excess
of that required for oxidative phosphorylation is available to further influence
subcellular constituents of living systems.

Several investigators have noted increased clonal growth of human diploid
fibroblasts at 1% compared with 20% oxygen. Richter et al. (10) reported
increased clonal growth of WI-38 cells incubated at 1% compared with 20%
oxygen. Packer and Fuehr (11) noted that cells seeded at 10^2 cells/cm^2 grew
better at 2 and 10% O_2 than at 20% O_2, but he detected no differences between
2 and 20% O_2 at a seeding density of 10^4 cells/cm^2. Taylor et al. (12) have
reported the only other study quantitating the density-dependent effect of
oxygen on mammalian fibroblasts in culture (12). They compared the growth of
cultures gassed with 1 and 18% O_2. The measured medium P_{O_2} of their cultures
gassed with 1% O_2 was 40–60 mm Hg, and of cultures gassed with 18% O_2,
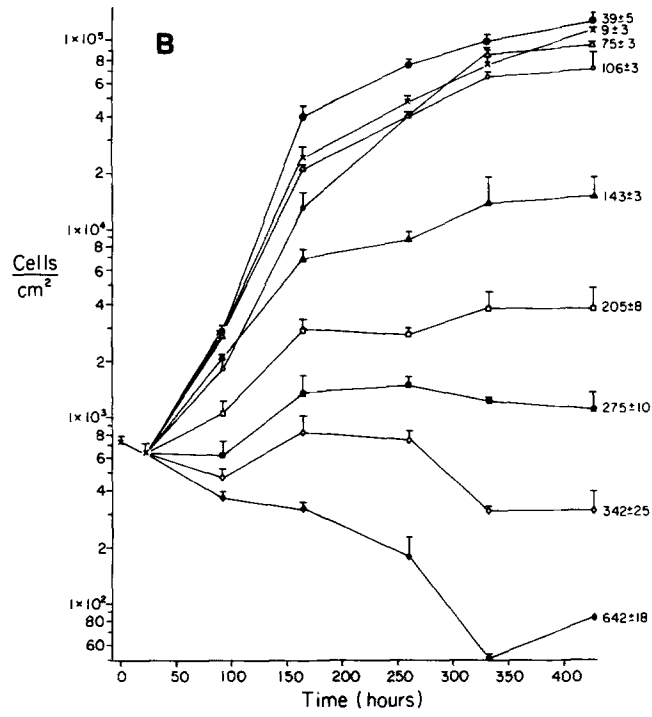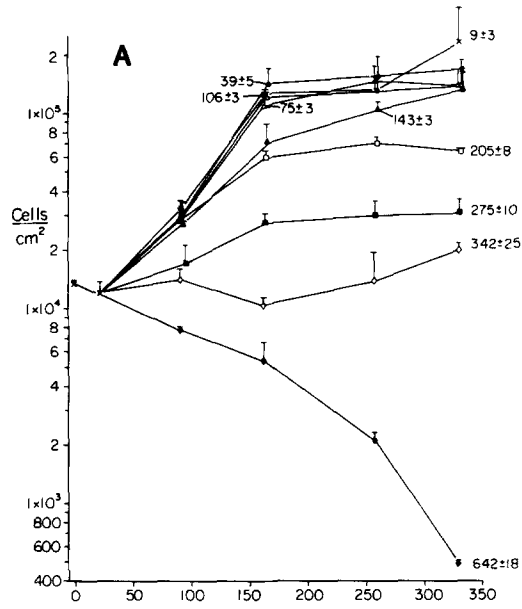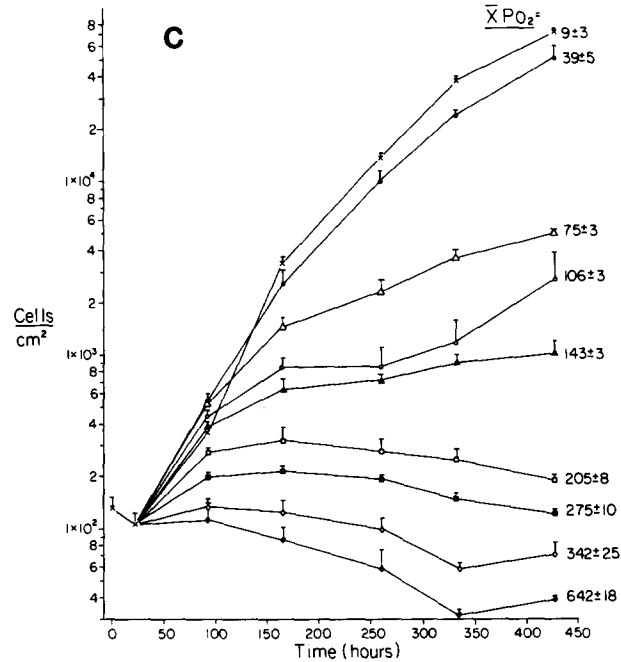
FIGURE 6. (A–C) Effect of oxygen tension on the growth of human diploid fibroblasts on gas-permeable membranes inoculated at different cell densities. Mid-passage IMR90 cells (91% labeled nuclei) were seeded into 20-cm² Petriperm dishes that have a hydrophilic 25-μm Teflon membrane as a growth surface. This membrane is permeable to oxygen and carbon dioxide. Dishes were placed into one of nine incubators (37°C) capable of maintaining a preselected oxygen concentration by an electronic feedback mechanism coupled to a Clark oxygen electrode within the incubator. 0.5 ml of media (DME + 10% fetal calf serum) was used per cm² of growth area. Numbers associated with each line represent the average partial pressure of oxygen of gas samples withdrawn from the incubator daily during the period of incubation and measured with a blood gas analyzer. Each point indicates the mean of triplicate dishes. The error bars indicate the standard deviation. Cells inoculated at (A) 10⁴ cells/cm², (B) 10³, (C) 10².

125–135 mm Hg. This emphasizes the necessity of measuring the actual PO_2 in each flask when studying the effect of oxygen on cell growth. Taylor et al. (12) reported better clonal growth of fibroblasts incubated at 40–60 mm Hg than at 130 mm Hg, but no difference in growth at these oxygen tensions when cells were seeded at 6,666 cells/cm².

Ambient air has a PO_2 of 158, tracheal air in the human, 149, alveolar air, 100, arterial blood, 95, and mixed venous blood, 40 (18). The PO_2 of venous blood exceeds the tissue PO_2 because gradients on the order of tens of millimeters of mercury probably exist between the blood in the capillary systems and the sites of oxygen reduction (19). The PO_2 of the basal layer of the epidermis is ~20 mm Hg when the ambient temperature is 20°C (20). The PO_2 of adult cerebral cortex of rats, mice, and guinea pigs is between 2 and 5 mm Hg (21). Oxygen is not uniformly available throughout the brain, and oxygen tensions varying 30–40 mm Hg exist within a distance of a few microns, although the PO_2 at a given microarea of cerebral tissue appears to be relatively constant (22). Thus the

effect we have observed in vitro on cellular growth of human fibroblasts occurs within a range of oxygen tensions normally found in mammals.

Our demonstration that growth is modulated by low partial pressures of oxygen supports the idea that oxygen may play a role in regulating the growth of cells in embryonic development. These effects may involve both the rate of growth and cellular differentiation. Caplan and Koutroupas (2) have suggested that regions of low oxygen tension promote development of cartilage cells, while regions of high oxygen tensions promote development of muscle cells in the limb of a developing chick.

The demonstration that low oxygen tensions enhance fibroblast growth points out the potential importance of hypoxia as a regulatory factor in wound healing. Wounds tend to be relatively hypoxic. Recently, Knighton et al. (23) showed that macrophages exposed to low oxygen tension elaborate an angiogenesis factor that stimulates blood vessel growth but is not mitogenic for fibroblasts. They suggest that hypoxia regulates some aspects of wound healing through elaboration of this factor. Our work shows that hypoxia per se promotes fibroblastic proliferation, in the absence of any other cell type.

In our previous work (7–9) we found that growth inhibition at elevated oxygen tensions occurs by different mechanisms, depending on the level of oxygen tension to which cells are exposed. The data presented here suggest that oxygen modulates cell growth at low oxygen tensions by a different mechanism than that seen under growth at P_{O_2} 350–600 mm Hg. Growth inhibition at $P_{O_2} > 350$ mm Hg appears to be independent of seeding density. Further studies are needed to determine where in the cell cycle low density cells are inhibited.

The mechanism is unknown by which higher seeding densities overcome oxygen toxicity within the physiologic range. Cells might secrete a growth-promoting factor into the medium that would enable them to bypass an essential process that is inhibited by oxygen at the higher end of the physiologic range. Medium from high density cultures might accumulate such putative material and become more resistant to oxygen. This hypothesis is supported by our observation that cell growth was consistently better when we used medium conditioned by cells that were growth inhibited at 10,000 cells/cm^2 by 95% O_2 for 1 wk compared with growth in medium incubated at 95% O_2 for 1 wk without cells. The density-dependent nature of the cells' ability to ameliorate the toxic effects of oxygen may be due to a density dependence in the ability of the cells to effectively scavenge reactive oxygen radicals. This could be caused by a density dependence in the ability of cells to induce intracellular antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase. Further studies to investigate these hypotheses are in progress.

Summary

We have examined the growth of human diploid fibroblasts (WI-38 and IMR90) as a function of initial seeding density and oxygen tension. Cells at young and mid-passage levels were subcultivated in Dulbecco's modified Eagle's medium with 10% fetal bovine serum at 0.005, 0.01, 0.03, 0.1, 0.3, 1, and 2 × 10^4 cells/cm^2. Flasks were equilibrated before and after seeding with 1 of 10 gas mixtures containing the desired oxygen tension (9–591 mm Hg) and placed in

incubators that measure and maintain a preset oxygen tension. The partial pressure of oxygen (Po_2) in media of all flasks was determined at harvest. Cells were shielded from light of wavelength <500 nm.

Cell growth varied inversely with oxygen tension and seeding density. At 50 cells/cm^2, growth was maximal at Po_2 9 and 16 mm Hg. Growth was progressively inhibited as the oxygen tension was increased. The population doubling increase at 14 d was 8.6 for Po_2 9 and 16 mm Hg, 5.8 for Po_2 42 mm Hg, 3.8 for Po_2 78 mm Hg, 3.8 for Po_2 104 mm Hg, and 3 for Po_2 138 mm Hg. As the seeding density was increased, the differences in growth at Po_2 <140 mm Hg were progressively minimized, such that at seeding densities of 10^4 cells/cm^2 there was little difference in the rate of exponential growth or the final saturation density of cells cultivated between Po_2 9 and 96 mm Hg. At all seeding densities tested, growth was progressively inhibited when the Po_2 was increased >140 mm Hg. The seeding density dependence of oxygen's influence on cellular growth is not explained by oxygen consumption of higher density cultures. Oxygen acts directly on the cells and not by destroying some essential medium component. We have found that oxygen regulates the growth of human cells under pressures of oxygen physiologic to humans, and that oxygen toxicity contributes to the seeding density dependence of cellular growth commonly seen in cell culture.

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