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A method for the long-term cultivation of mammalian cells in the absence of oxygen: Characterization of cell replication, hypoxia-inducible factor expression and reactive oxygen species production

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ABSTRACT

The center of tumors, stem cell niches and mucosal surfaces all represent areas of the body that are reported to be anoxic. However, long-term study of anoxic cell physiology is hindered by the lack of a sustainable method permitting cell cultivation in the complete absence of oxygen. A novel methodology was developed that enabled anoxic cell cultivation (17d maximum time tested) and cell passage. In the absence of oxygen, cell morphology is significantly altered. All cells tested exhibited morphologic changes, *i.e.*, a combination of tethered (monolayer-like) and runagate (suspension-like) morphologies. Both morphologies replicated (Vero and HeLa cells tested) and could be passaged anaerobically. In the absence of exogenous oxygen, anoxic cells produced reactive oxygen species (ROS). Anaerobic runagate HeLa and Vero cells increased ROS production from day 3 to day 10 by 2- and 3-fold, respectively. In contrast, anoxic tethered HeLa and Vero cells either showed no significant change in ROS production between days 3 and 10 or exhibited a 3-fold decrease in ROS, respectively. Detection of ROS was inversely related to detection of hypoxia-inducible factor-1α (*HIF1*) mRNA and HIF-1 protein expression which cycled over a 10-day period. This methodology has broad applications for the study of tumor and stem cell physiology as well as gastrointestinal cell-microbiome interactions. In addition, sustainable anaerobic cell culture may lead to the identification of novel pathways and targets for chemotherapeutic drug development.

1. Introduction

The effects of hypoxia on mammalian cell physiology are the focus of numerous studies (Glover and Colgan, 2011; Gronroos et al., 2014; Karhausen et al., 2005; Semenza, 2000; Semenza, 2016). However, substantial areas of both human and animal bodies are anoxic (no oxygen) under normal and pathophysiological conditions (Biedermann and Rogler, 2015; Cui et al., 2013; Grayson et al., 2007; Mohyeldin et al., 2010). These areas include mucosal surfaces, the center of tumors, and stem cell niches. Under normal physiologic conditions, the intestinal epithelium experiences oxygen fluctuations which range from severely hypoxic to anoxic (Biedermann and Rogler, 2015; Glover and Colgan, 2011). In analogous findings, stem cell niches contain areas of anoxia (Ivanovic, 2009; Mohyeldin et al., 2010). This sensitivity of stem cells to oxygen also extends to *in vitro* stem cell cultivation wherein routine *in vitro* cultivation is optimal under hypoxic conditions (Cui et al., 2013; Liao et al., 2008; Park et al., 2008; Semenza, 2000; Semenza, 2016). This inherent ability of mammalian cells to survive in anoxic conditions is further substantiated by whole animal studies which show that naked mole rats can survive 18 min without oxygen by switching from a glucose to fructose based metabolism (Park et al., 2017).

Beyond anoxia's role in normal physiology, the centers of solid tumors, *e.g.* breast and prostate, contain anoxic regions (Alimirah et al., 2006; Lawson et al., 2009), which are attributed to the relatively avascular tumor microenvironment and poor oxygen perfusion (Döme et al., 2007). The anoxic character of tumor centers is independently validated by the colonization of solid tumor centers by obligate anaerobic bacteria (Cummins and Tangney, 2013).

These *in vivo* observations of consistent anoxic mammalian cell growth have not been supported by anoxic *ex vivo* studies. Although some studies have cultivated mammalian cells under anaerobic conditions, these studies are terminated around day three. Study termination is typically due to reported loss of cell viability, alteration in cell morphology (cell rounding), and cell detachment from solid substrate (Fack et al., 2015; Lam et al., 2009; Schroedl et al., 2002). Taken

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together, these general observations result in an acceptance of the notion that mammalian tissue culture cells don't survive for more than a few days in an anaerobic environment (Fack et al., 2015; Lam et al., 2009; Schroedl, et al., 2002). However, this convention is antithetical to the fluctuations in oxygen levels from anoxic to normoxic under which multicellular life evolved and the most recent findings in studies on naked mole rat physiology (John and Whatley, 1975; Martin et al., 2015; Park et al., 2017).

Phylogenetic evidence indicates that cellular mitochondria evolved in parallel with bacterial evolution from the Universal Common Ancestor of the Tree of Life (Gray, 2017; Harish and Kurland, 2017; Lane, 2017; Martin, 2017). Before oxygenation of the oceans (about 580 million years ago), it is estimated that mitochondrial life spent 1. 542 million years in anoxia. Thus, it is reasonable that, like their bacterial counterparts, mammalian cell precursors developed pathways for anaerobic respiration that utilized alternative electron acceptors (Arai et al., 1998; Müller et al., 2012). The continued presence of these alternative respiratory systems is proven, in part, by the ability of isolated mitochondria to produce ATP and reactive oxygen species (ROS) under anaerobic conditions (3 hrs under anoxic conditions) using nitrite and TCA cycle intermediates (Gupta and Igamberdiev, 2011, 2016; Kozlov et al., 1999; Nohl et al., 2005; Stoimenova et al., 2007). However, the question remains as to whether anaerobic respiratory pathways, with a non-oxygen terminal electron acceptor, function in intact cells to support fundamental physiologic processes. This study describes a straightforward accessible methodology for the long-term cultivation of mammalian cell lines under anoxic conditions and describes cell characteristics in the absence of oxygen.

2. Material and methods

2.1. Cell culture

2.1.1. Cell line maintenance

HeLa 229 - human cervical epithelial, Hep2 - HeLa cell derivative, L929 - murine fibroblast, Vero - Monkey kidney epithelial, CHO - Chinese hamster ovary epithelial, HT22 - murine hippocampal neuronal cells, MCF-7 - human breast epithelial carcinoma, MDA-MB-231 - human triple neg. breast epithelial carcinoma, and DU 145 - human prostate epithelial carcinoma cell lines were used in this study. The cell lines were generously provided by Drs. Fay, Ramsey, Tiwari and Malaiyandi, Midwestern University. Cell lines were maintained under normoxic conditions (5% CO_2 in air; 37 °C) in high glucose (4.5 g/L) DMEM medium with pyruvate and glutamine (584 and 110 mg/L, respectively; Cellgro) that was supplemented with 10% fetal bovine serum (FBS) and gentamicin (HyClone, Logan Utah, USA).

2.1.2. Anoxic cell culture

A novel medium (PS-74656), which was developed for this project, supported the anaerobic cultivation of all cells lines with the exception of MDA-MB-231. PS-74656 medium is a standard low glucose (1 g/L) DMEM medium with pyruvate and glutamine (584 and 110 mg/L, respectively; Cellgro) that was supplemented with 10% fetal bovine serum (FBS; VWR International) and 50 mM nitrite. MDA-MB-231 cell line required an alternative formulation (PS-74656-A) for anoxic growth. PS-74656-A was a standard high glucose (4.5 g/L) DMEM medium supplemented with pyruvate and glutamine (584 and 110 mg/L, respectively; Cellgro) 10% FBS, 10 mM nitrite and 0.1 mM glucosamine. Of note is that 20% FBS was toxic under anaerobic, but not normoxic conditions for Vero and HeLa cells (only cell lines tested). Initiation of anoxic culture occurs over a 2-day period, *i.e.*, day (-)1 and day 0. Day 1 for all determinations was defined as 24hr post day 0. On designated day (-)1, cells were seeded at 2.24×10^5 cells/well in 24 well plates, which corresponds to 80% confluency, in the high glucose DMEM, described above. Cell plates *vs.* flasks were used throughout to maximize gas exchange and minimize to the greatest extent possible introduction of oxygen into the system while maintaining sterility. The maximum cell concentration used was 80% confluency (2.24×10^5 cells/well in 24 well plates or 1.12×10^6 cells/well in 6 well plates). These monolayers were incubated overnight under normoxic conditions (5% CO_2, 37 °C; 16–24hr normoxic incubation) to allow for establishment of cell monolayers. At day 0, the monolayers were transferred to the anaerobic chamber (Whitney A35, anaerobic gas mixture H_2, CO_2, N_2; 37 °C). The aerobic medium was immediately removed and replaced with degassed PS-74656 medium or PS-74656-A, as appropriate. Degassed medium was prepared by incubation at room temperature for 48hr under vacuum (20 mL medium in 50 mL conical centrifuge tubes; 45° angle), or until all gas bubbles disappeared. The medium was then acclimated for 24 hr to anoxic gases (hydrogen/carbon dioxide/nitrogen). All transfer pipets, tissue culture plates and pipet tips were also preincubated in the anaerobic chamber and flushed with anaerobic gas prior to use. The lack of oxygen in degassed PS-74656 medium was confirmed by monitoring it with an oxygen electrode (Microelectrodes, Inc. oxygen probe; Pod-Vu software). The oxygen levels on day 0 in the cultures with degassed medium was consistently 0.3% oxygen. By day 1 (24hr post-placement of cell monolayers in anaerobic chamber with degassed medium), the oxygen levels in test cultures were undetectable (0% oxygen) which constitutes our definition of anoxia, given the limitation of the instrumental and experimental parameters. Controls consisted of parallel normoxic cells from the same cell lot which were used for anoxic culture. Normoxic controls were handled as described for anoxic cells with the exception that at day 0, atmospheric PS-74656 or PS-74656-A media were used for normoxic incubation (5% CO_2, 37 °C) and all cell manipulations were performed under normal atmospheric oxygen levels.

2.2. Cell longevity determination

Anoxic culture viability was monitored at 18–24hr intervals by phase contrast microscopy. The 24 or 6 well plates were placed in ziplock bags, that had been flushed and filled with anaerobic gas. These sealed bags were then removed from the anaerobic chamber for phase contrast microscopy (200x and 400x initial magnification) and photomicrographs obtained. Immediately after the photomicrographs were obtained, cells in suspension (runagate cells) were removed (700 µL) and centrifuged (10 min; 2000 rpm; room temperature). After centrifugation, the cell supernatants (600 µL) were tested for levels of lactate dehydrogenase (LDH) (Ponsoda et al., 1991). Relative levels of LDH over time were used as a measure of cytotoxicity. These were determined using the supernatant for all cell lines, controls, and test conditions after incubation. The amount of color change was measured at an absorbance of 450 nm (Thermo-Fisher Multiskan FC). Cells in the monolayer (tethered cells) were trypsinized (150 µL/well of 24 well plate, 0.05% trypsin with 0.53 mM EDTA, Corning). Trypsin was neutralized with medium containing 10% FBS (1 mL) and cells were removed. All cell samples were then diluted in trypan blue for cell counting and viability testing (Bio-Rad TC20 cell counter). Controls consisted of cells from the same lot processed in parallel but incubated under normoxic standard conditions (5% CO_2 in air; 37 °C).

2.3. Actin staining

For actin visualization, CytoPainter Phallodian-iFluor 555 (Abcam) was used according to manufacturer's recommendations. Aerobic and anaerobic adherent cells (monolayer and tethered, respectively) and non-adherent cells (suspension and runagate, respectively) were grown as described. Cells were fixed and permeabilized with 3% paraformaldehyde in PBS with 1% bovine serum albumin and 0.1% Triton X-100 (Sigma-Aldrich). Cells were stained with the phalloidin-ifluor for 45 min. After four washes, the cells were counterstained with Nuclear Green (Abcam) which was prepared and used according to manufacturer's recommendation. Fluorescent microscopy was performed on