Gaseous Signaling Compounds (Hydrogen Sulfide and Nitric Oxide) and Their Relative Roles in Affecting Anaerobic HeLa 229 Cell Viability

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Abstract: Metabolic pathways supporting long-term anaerobic cell viability have not been identified. The effect NO and H₂S pathway effectors have on HeLa 229 cell viability was measured after 10 days anaerobic incubation. The addition of arginine or xanthine (NO pathway precursors) consistently increased HeLa cell viability by 13.1- and 4.4-fold, respectively. Allopurinol, a xanthine oxidase inhibitor, also increased viability, as compared to control levels. In contrast, inhibition of iNOS by 1400W increased cell viability by 79-fold. Regarding the H₂S pathway, precursor cysteine enhanced viability by 9.8-fold with the greatest number of viable cells measured in response to the presence of a H₂S donor (GYY4137), or an inhibitor of glutathione synthesis, propargylglycine (40- and 85-fold, respectively). These results demonstrate that the constitutive level of cell viability after extended (10 days) growth without oxygen can be modulated by affecting NO or H₂S generating pathways.

Keywords: cysteine; hydrogen sulfide; arginine; xanthine; nitric oxide; hypoxia inducible factor (HIF); anoxia.

1. Introduction

A majority of studies on tumor cell physiology are performed under carbon dioxide enriched atmospheric oxygen conditions. However, this oxygen-rich environment is not physiologically relevant. *In vivo*, the ecosystem ranges from completely lacking in oxygen (anoxic/anaerobic) to hypoxic (less than 13%) (Cojoc et al., 2015; Ivanovic, 2009). While there has been a push towards characterizing cell metabolism under physiologically relevant oxygen levels (physioxia), cell metabolism as it relates to growth under strict anoxic microenvironmental conditions has not been studied. To date, assays performed in an anaerobic macroenvironment are typically initiated using media containing atmospheric oxygen levels that deplete over time; thus, yielding incubation conditions that cover an oxygen concentration spectrum, the nadir of which is not measured (Vlaski-Lafarge et al., 2020).

Characterization of anaerobic cell growth is crucial, particularly since like stem cells in their niches, solid tumors also contain areas lacking an oxygen supply (anaerobic) (Cummins and Tangney, 2013; Gronroos et al., 2014; Visvader and Lindeman, 2008). It is essential to characterize this aspect of cancer cell metabolism to fully understand metastasis and drug resistance. Until recently, the ability to study long-term anoxic cell growth has been elusive, due to an inability to culture cells *ex vivo* for extended periods of time in the absence of oxygen. We pioneered a system that mimics the lack of oxygen in tumor centers from where cancer stem cells, and potentially metastatic cells, are reported to originate. *In vitro* support of the role anaerobic rewiring may enhance tumorigenesis is the finding that HeLa cells grown anaerobically shift their cytokine secretome expression to one that is pro-angiogenic (Plotkin et al., 2018b). The pathways that play a role in long-term anaerobic cell viability have not been identified. The gaseous intercellular transmitters e.g., nitric oxide (NO) and hydrogen sulfide (H₂S) serve critical roles in tumor microenvironments and acquired chemotherapy resistance (Chen et al., 2019; Cheng et al., 2014; De Vries and Schröder, 2002; Szabo et al., 2013). Since bacteria, which undergo anaerobic respiration, use NO and H₂S as components of their respiratory pathways, we focused on determining whether NO and H₂S generating pathways play a role in sustaining anaerobic cancer cell viability, as an index of metabolism (Pal et al., 2018; Plotkin et al., 2015; Stoimenova et al., 2007; Trageser and Uden, 1989).

2. Materials and Methods

Reagents. Effectors for nitric oxide synthesis used in experiments were S-nitroso-N-acetylpenicillamine (SNAP; 50μM; Cayman Chemical), arginine (10mM; Sigma), N omega-nitro-L-arginine methyl ester hydrochloride (L-NAME; 0.15mM; Sigma), N-([3-(aminomethyl)phenyl] methyl) ethanimidamide dihydrochloride (1400W; 100μM; Sigma), xanthine (4x10⁻⁵M; Sigma), nitrite (50, 100, 500mM; Sigma), and allopurinol (200μM; Sigma). Effectors for hydrogen sulfide synthesis used in experiments were cysteine (0.4mM; Sigma), JK-2 (300μM; Sigma), P-(4-Methoxyphenyl)-P-4-morpholinyl-phosphinodithioic acid (GYY4137; 400μM; Cayman Chemical), pyridoxal phosphate (PLP; 2μM; Sigma), and DL-propargylglycine (PAG; 0.5mM; Sigma).

Anoxic Cell Culture. Previous studies show that regardless of cell line screened, all can replicate in the absence of oxygen (Plotkin et al., 2018a). For this initial study, to assess the roles various pathways and their mediators play in supporting anaerobic cell viability, HeLa 229 cells were used based on their replication rate and consistency as the model cell line for initial determinations of anaerobic metabolic changes. HeLa 229 cells were grown to 80% confluence (2.24 x 10⁵ cells/well) in 24 well plates (normoxic conditions, 5% CO₂ in air; 10% FBS, 0.05mg/ml gentamicin (Hyclone); high glucose (4.5 g/L) DMEM

medium with glutamine and pyruvate (584 and 110 mg/L, respectively; Cellgro) for 24h. These cells were then transferred to an anaerobic chamber (Whitley A35, anaerobic gas mixture: H₂, CO₂, N₂; 37°C), and medium replaced with degassed low glucose (1 g/L) homologous medium without gentamicin, as previously described (Plotkin et al., 2018a). The lack of oxygen (0%) in the degassed medium and culture plate wells were confirmed by oxygen electrode measurements (Microelectrodes, Inc. oxygen probe; Pod-Vu software).

Nitric Oxide and Hydrogen Sulfide Pathway Constituents and Selected Inhibitors. Nitric oxide and hydrogen sulfide producing pathways could participate in supporting anaerobic mitochondrial respiration. To test this hypothesis, precursors, inhibitors, and agonists of these pathways were tested for their effect on cell viability and hypoxia inducible factor (HIF) expression. Test chemicals were dissolved in degassed medium under anaerobic conditions. Upon transferring the cells to the anaerobic chamber (Day 0), aerobic DMEM was removed by aspiration and replaced with anaerobic degassed DMEM alone, or containing chemical supplement indicated. At Day 10, the total number of viable cells per condition were determined by trypan blue dye exclusion (Countess™ II Automated Cell Counter) and HIF expression measured (see below). Controls for all tests consisted of cells grown in anaerobic medium alone. Medium with DMSO (0.05%) was used as the control for chemicals requiring DMSO for solution preparation (allopurinol, 1400W, and GYY4137).

HIF-1α Analysis. HIF expression for each growth condition was determined. Immediately, post-viability measurements were taken, plate well contents (on ice) from each condition were pooled for six wells (4°C), then centrifuged (2000 RPM, 5 min, 4°C). Supernatants were removed. HIF-1α protein was extracted from cell pellets and confirmed using Human Simple Step ELISA kit (Abcam) according to manufacturer's instructions.

Data Analysis. Data were analyzed (GraphPad Prism) by two-way ANOVA ($p \leq 0.05$). Where appropriate, Tukey post-hoc tests were performed. Significant points ($p < 0.05$) are designated with an asterisk and standard error of the mean (SEM) are included on all graphs.

3. Results and Discussion

In solid tumors, there are intra-tumor microenvironments that are anaerobic. As previously reported, replicating HeLa cells at 10 days anaerobic incubation produce reactive oxygen species (ROS), as detected by DCFDA and CellRox Green fluorescence, lack expression of the autophagy marker LC3B and accumulate MitoTracker Red FM (Plotkin et al., 2018a). Two important pathways that result in NO

release are the oxidation of L-arginine by nitric oxide synthase (NOS), and the nitrite reductase pathway (xanthine oxidase, XO) that can utilize either xanthine, or nitrite as a precursor (Cantu-Medellin and Kelley, 2013; Damacena-Angelis et al., 2017). In bacteria and plants, nitrite can fuel ATP production via the mitochondrial electron transport chain with NO as the terminal electron acceptor (Gladwin et al., 2005; Siva Raju et al., 1997; Stoimenova et al., 2007). In mammalian cells, XO oxidizes either xanthine or nitrite to form NO. The ability of nitric oxide generating pathway constituents to support long-term (10 days) anaerobic cell viability was determined (Fig. 1A; 1B). Of the three fundamental precursors, the addition of xanthine and arginine resulted in a reproducible pattern of increased cell viability by 13- and 4.4-fold, respectively, as compared to supplement-free control, with no associated increase in HIF expression (Fig. 1C). Allopurinol, an inhibitor of xanthine oxidase, resulted in only a 3-fold change in cell viability, while inhibition of the constitutive NOS (cNOS) by L-NAME had no effect on cell viability as compared to control. In contrast, the combination of allopurinol and L-NAME enhanced cell viability by 97-fold, as did the addition of the iNOS inhibitor 1400W alone, and when combined with L-NAME (80-fold and 103-fold increase, respectively). All other supplements tested either had no effect on constitutive anaerobic cell viability, or increased viability except for SNAP, a nitric oxide donor, which depressed viability levels to 0.6-fold of control. Thus, with respect to NO pathways, inhibition of NO production via 1400W and allopurinol (xanthine oxidase) had the most robust effect on increasing cell viability. Another interesting finding relative to anaerobic metabolism that differentiates from what is reported for cell metabolism under hypoxic conditions is that the change in viability appears disconnected from HIF expression. Except for the addition of nitrite (alone or in combination), HIF protein levels were depressed as compared to medium control. Of the supplements tested, only the presence of nitrite significantly enhanced HIF expression, as would be predicted based on reports that nitrite helps stabilize HIF (Diwakar and Ravindranath, 2007) (Fig 1C).

Like NO generation, multiple parallel pathways are involved in H₂S synthesis from the precursor cysteine (Fu et al., 2012). These include the cystathionine-β-synthase (CBS) branch, located in the central nervous system; the cystathionine γ-lyase (CSE) branch, located in the vasculature, liver, and kidney; and the 3-mercaptopyruvate sulfur transferase (3-MST) branch, which is found in kidney, liver, lung, heart, muscle, spleen, and brain (Nagahara et al., 1998). H₂S oxidation, which occurs widely throughout eukaryotes, is coupled to ATP synthesis by a mitochondrial electron transport chain through a sulfide quinone oxidoreductase (Giuffrè and Vicente, 2018; Yong and Searcy, 2001). H₂S also regulates expression of cytochrome c oxidase (Guo et al., 2012; Murphy et al., 2019; Vicente et al., 2016). Interestingly, the cysteine/CSE/H₂S pathway is involved in melanoma progression, and has been shown to promote cell

cycle progression in squamous cell (oral) carcinoma cells *in vitro* (Bhattacharyya et al., 2013; Chakraborty et al., 2018; Ma et al., 2015; Panza et al., 2014).

Overall, any treatment that increased cellular H₂S levels, particularly the slow H₂S donor GYY4137, or the inhibition of CSE, which blocked glutathione, a consumer of H₂S synthesis, caused significantly ($p < 0.05$) enhanced viability, as compared to medium alone (cysteine, 9.8-; JK-2, 8.8-; and GYY4137, 40.4-fold increases; Fig 2A). This finding is in contrast to reported negative effects on viability when CSE expression (siRNA) is silenced in the presence of oxygen, or after exposure to an oxidizing agent (hydrogen peroxide) (Lee et al., 2014). Since under anaerobic culture there is minimal need for the antioxidative properties of H₂S to combat cellular reactive oxygen species generated oxidative stress, the predictably higher H₂S levels could be used to drive mitochondrial ATP production (Fu et al., 2012; Plotkin et al., 2018a; Szabo et al., 2013). Interestingly, except for nitrite which has been reported to affect HIF expression, HIF expression lacked correlation with cell viability under anaerobic growth conditions. In addition, H₂S had no significant effect on HIF expression, contrary to its effect in *Caenorhabditis elegans* (Fig 1C; 2C) (Budde and Roth, 2010). This finding casts doubt on HIF's relevance for cells growing long-term anaerobically, in contrast to its importance in hypoxically-grown cells (Singh et al., 2012).

Depending on the organ system, and type of NO synthase, H₂S exhibits contradictory effects. These effects include inhibition of NO produced by the neuronal form of nitric oxide synthase (nNOS), or increased NO production by iNOS and eNOS (Heine et al., 2015; Hou et al., 2017). In addition, H₂S appears to function as a feedback inhibitor of iNOS and nNOS, presumably due to its function as a free radical scavenger (Kolluru et al., 2013; Kolluru et al., 2015). To determine if constituents of H₂S producing pathways support HeLa cell anaerobic viability, cysteine, H₂S donors JK-2 (short term) and GYY4137 (slow release), pathway cofactor pyridoxal phosphate (vitamin B6), or propargylglycine (PAG), the irreversible inhibitor of cystathionine γ -lyase and subsequent glutathione synthesis, were tested (Fig. 2A).

All H₂S-related supplements tested increased viability (Fig 2A). The most potent enhancers of cell viability were the H₂S donors GYY4137 and PAG (Diwakar and Ravindranath, 2007; Fu et al., 2012; Lee et al., 2011). The effects for cysteine and vitamin B6 ranged from 10- and 19-fold increase, respectively. As was measured for nitric oxide pathways, nitrite was the sole supplement responsible for increasing HIF expression compared to control (Fig 2B). Interestingly, the level of HIF in cells grown with cysteine alone and with its cofactor vitamin B6 was 30% and 11% that of cells grown in control medium, respectively,

indicating that factors that lead to H₂S production further destabilize HIF protein in cells incubated for extended periods (10 days) in the absence of oxygen. This is the first report of the effects various gaseous chemical signaling molecules, and their inhibitors, have on the viability of anaerobically grown cells, and the role H₂S plays in sustaining viability in the absence of oxygen. Further studies on anaerobic mitochondrial function are ongoing.

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Fig. 1. Anaerobic HeLa cell viability modulation by mediators of the nitric oxide generating pathways. (A,B) Number of viable cells/well present after 10 days of anaerobic incubation with various mediators of the pathways responsible for nitric oxide generation. Treatments (Mean ± SEM; n=6, repeated once to twice); * = $p < 0.05$ considered significant; (C) Hypoxia inducible factor (HIF) expression by HeLa cells grown anaerobically in the presence of various mediators which promote, or inhibit, the generation of nitric oxide. Each HIF measurement is a pooled sample from viability measurements. HIF protein level (Mean ± SEM; n=6, repeated once to twice); * = $p < 0.05$ considered significant.

Fig. 2. Anaerobic HeLa cell viability modulation by mediators of the hydrogen sulfide pathways. **(A)** Number of viable cells/well present after 10 days of incubation with various mediators of the hydrogen sulfide pathways under anaerobic conditions; **(B)** Expression of hypoxia inducible factor (HIF) response to the various mediators after growth under anaerobic conditions. Each HIF measurement is a pooled sample from viability measurements. Mediators and HIF (Mean $\pm$ SEM; n=6, repeated once to twice); * = $p < 0.05$ considered significantly different from control.

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