Flux Analysis Shows That Hypoxia-Inducible-Factor-1-Alpha Minimally Affects Intracellular Metabolism in Tumor Spheroids

Byoung-jin Kim, Neil S. Forbes

Department of Chemical Engineering, University of Massachusetts, Amherst, 686 North Pleasant Street, Amherst, Massachusetts 01003-9303; telephone: (413) 577-0132; fax: (413) 545-1647; e-mail: forbes@ecs.umass.edu

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ABSTRACT: Heterogeneous metabolic microenvironments in tumors affect local cell growth, survival, and overall therapeutic efficacy. Hypoxia-inducible-factor-1α (HIF-1α) is a transcription factor that responds to low-oxygen environments by upregulating genes for cell survival and metabolism. To date, the metabolic effects of HIF-1α in three-dimensional tissue have not been investigated. Preliminary experiments have shown that the effects of HIF-1α are dependent on glucose availability. Based on this observation, we hypothesized that HIF-1α would not affect cell survival and metabolism in the center of spheroids, where the concentrations of oxygen and glucose are low, similar to hypoxic regions found in tumors. To test this hypothesis we used fluorescence microscopy and the tumor cylindroid model to quantify cellular viability in three-dimensional tissue. Isotope labeling and metabolic flux analysis were also used to quantify the intracellular metabolism of wild-type and HIF-1α-null spheroids. As hypothesized, cell survival and intracellular metabolism were not different between wild-type and HIF-1α-null tissues. In addition, small spheroids, which contain less quiescent cells and are less nutritionally limited, were found to have increased carbon flux through the biosynthetic pentose phosphate and pyruvate carboxylase pathways. These results show how nutrient gradients affect cell growth and metabolism in spheroids and suggest that metabolic microenvironment should be taken into account when developing HIF-1α-based therapies.

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KEYWORDS: metabolic flux analysis; tumor; hypoxia-inducible factor; HIF-1α; metabolism; spheroid; biosynthesis

Introduction

Solid human tumors contain heterogeneous populations of cells, which can be divided into three broad types: proliferating, quiescent, and necrotic (Sutherland, 1988; Sutherland and Durand, 1984). These cellular subpopulations arise because of the chaotic vasculature in tumors (Jain, 1998; Vaupel et al., 1989) which limit the supply of nutrients, wastes, and growth factors (Freyer and Sutherland, 1986; Sutherland, 1988; Venkatasubramanian et al., 2006). Heterogeneity in tumors reduces the efficacy of cancer therapy: chemotherapeutics are less effective against quiescent cells (Tannock et al., 2002) and radiation therapy requires oxygen to effectively kill cancer cells (Kumar, 2000; Molls et al., 1998; Vaupel et al., 2001). Spheroids are a useful model of the microenvironments in tumors because they contain the same nutrient gradients and cell types as tumors (Sutherland, 1988; Sutherland and Durand, 1984). In addition, quiescent cells in spheroids have been shown to have reduced volume, mitochondrial function, and oxygen uptake compared to the proliferating cells in spheroids (Bauer et al., 1982; Freyer and Sutherland, 1980).

Hypoxia, or a physiologically low-oxygen concentration, is one of the major factors responsible for the formation of quiescence and necrosis in tumors and three-dimensional cultures (Freyer and Sutherland, 1986). The presence of hypoxia in tumors is correlated with poor patient prognosis (Brzel et al., 1997; Harris, 2002) and is a distinctive marker that can be used as a therapeutic target (Giaccia et al., 2003; Semenza, 2003; Unruh et al., 2003). Hypoxia-inducible factor-1α (HIF-1α) is a transcription factor that enables cells to sense their local oxygen concentration (Semenza and Wang, 1992; Wang and Semenza, 1995). The concentration of HIF-1α, which is constitutively expressed, is regulated by oxygen through ubiquitin-mediated degradation (Huang et al., 1998; Salceda and Caro, 1997) mediated by the Von Hippel-Lindau Factor (Cockman et al., 2000; Kamura et al., 2000; Ohh et al., 2000). HIF-1α controls the expression of...
Figure 1. Cellular effects of HIF-1α. The concentration of HIF-1α is regulated by the local oxygen concentration. HIF-1α is a transcription factor that induces the expression of genes for cell survival, apoptosis, angiogenesis, and glycolysis. In glycolysis, HIF-1α upregulates the expression of the following enzymes: glucose transmembrane transporter (GLUT), hexokinase (HK), phosphofructokinase (PFK), glyceraldehyde-phosphate-dehydrogenase (GAPDH), enolase (ENO), and lactate dehydrogenase (LDH).

many genes that allows cells to survive in a low-oxygen environment including genes for cell survival, apoptosis, angiogenesis (VEGF), and glycolysis (Fig. 1; Iyer et al., 1998; Semenza, 2001; Semenza, 2003). Through its effects on glycolysis, HIF-1α is part of the machinery that enables cells to switch from aerobic to anaerobic metabolism to allow cells to survive in the absence of oxygen, which is known as the Pasteur Effect (Seagroves et al., 2001). HIF-1α has also been proposed as a therapeutic target because of its role in tumor progression (Giacca et al., 2003; Pili and Donehower, 2003; Semenza, 2003) and because its overexpression has been correlated with higher patient mortality in many human cancers (Semenza, 2003). Some HIF-1α targeting drugs are now in clinical trials (Semenza, 2003).

Recently, multiple groups have developed HIF-1α-null cell lines (Carmeliet et al., 1998; Ryan et al., 2000). Much of our current understanding of the function of HIF-1α comes from growth of these cells in monolayer cultures (Seagroves et al., 2001) and as tumors in mice (Carmeliet et al., 1998; Ryan et al., 2000). Previously, Johnson and coworkers showed that HIF-1α-null cells do not survive as well as wild-type cells when grown in hypoxic monolayer cultures that are well supplied with glucose (Seagroves et al., 2001). They found, however, that this effect is not observed in the absence of glucose. In hypoxic environments that are low in glucose, the survival of wild-type and HIF-1α-null cells is equally poor (Seagroves et al., 2001). In tumors hypoxic regions often have low-glucose concentrations (Walenta et al., 2001), a phenomenon that is also seen in multicellular spheroids (Freyer and Sutherland, 1986; Walenta et al., 2000). These observations lead us to hypothesize that HIF-1α would not improve cell survival in the hypoxic region at the center of spheroids.

In this work we investigated the effects of HIF-1α on cell survival and intracellular metabolism in three-dimensional tumor spheroids. We used fluorescence microscopy and the tumor cylindroid model to quantify the effect of HIF-1α on cellular viability. Isotopic labeling and flux analysis were used to quantify the fluxes through the major metabolic pathways of the cells in spheroids. The effect of spheroid size on intracellular metabolism was also quantified to demonstrate how nutrient gradients in spheroids affects local cellular metabolism. Our results show that HIF-1α has little effect on the metabolism of cells grown in spheroids. However, the presence of nutrient-limited quiescent cells had a sizable effect on metabolism. These results describe how HIF-1α may operate in tumors, how macroscopic nutrient gradient affect localized cell behavior, and the limitations of HIF-1α-targeted cancer therapies.

Materials and Methods

Cell Culture

Wild-type (HIF-1α+/+) and nullizygous (HIF-1α−/−) cell lines were a kind gift from Dr. R. Johnson (University of California, San Diego, CA). Both lines are ras-transformed mouse embryonic fibroblasts derived from mouse embryonic stem cells. The wild-type and HIF-1α-null cells were created by flanking each allele of HIF-1α withloxP sites and infecting the cells with adenovirus expressing either β-galatosidase or cre-recombinase, respectively (Ryan et al., 2000). Cells were grown in high-glucose Dulbecco’s Modified Eagle’s Medium (DMEM, 4.5 g/L glucose) containing 25 mM HEPES buffer (pH 7.4) supplemented with 10% fetal bovine serum. All cultures were maintained at 37°C with 5% CO₂ in a humidified incubator.

Spheroid Culture

Spheroids were formed by inoculating a single-cell suspension into culture flasks coated with poly(2-hydroxyethyl methacrylate), which prevented cell adhesion to the flask surface. In coated flasks, embryonic fibroblast cells naturally formed into aggregates and symmetric spheroids. Larger volume cultures (500–1,000 mL) were grown by transferring spheroids from T-flasks into spinner flasks. Adhesion was prevented in spinner flasks by stirring slowly at 100 RPM. The medium was changed every 2 days by turning off the stirrer, allowing the spheroids to precipitate for a few minutes, and replacing 70% of the medium. The average spheroid size was dependent on the time in culture as described in the results.
Spheroid size was determined by acquiring microscope images of at least 25 spheroids suspended in drops (2–5 mL) of culture medium that were removed from well mixed T-flasks or spinner flasks. Diameters were measured by manually fitting ellipses to the spheroid boundaries and averaging the lengths of the major and minor axes.

The number of cells in spheroid cultures was determined by counting the number of cells in 1 mL of medium removed from well mixed T-flasks or spinner flasks. Samples were centrifuged and the medium was removed. After washing with 1 mL PBS, spheroids were treated with trypsin-EDTA for 10 min and dissociated by repeated pipetting. Complete media and trypan blue were added and the number of cells was measured by hemocytometer. The amount of trypsin-EDTA, complete media and trypan blue were varied depending on the size of the spheroids; the volume ratio of the three was maintained at 1:1:2.

Spheroid Size Distribution

The diameters of spheroids growing in spinner flasks were measured as a function of time to determine the effect of culture time on the spheroid size distribution. Three methacrylate-coated T-225 flasks were inoculated with $1.5 \times 10^5$ cells/mL. After 4 days, the spheroids were transferred into 100 mL spinner flasks. On days 4 and 9 after inoculation the sizes of the spheroids in the culture were measured as described above. Histograms were generated for 4- and 9-day cultures by grouping spheroids into 50 and 100 μm bands, respectively.

Spheroid Growth

Growth curves of spheroids composed of wild-type and HIF-1α-null cells were determined by inoculating single-cell suspensions into methacrylate-coated T25 flasks in triplicate at a density of $6.25 \times 10^4$ cells/mL. Flasks were used to determine spheroid growth because more parallel replicates could be performed than in spinner flasks. The medium in the flasks was replaced every 2 days. Spheroid diameter was measured on days 5, 9, 14, 17, and 21, as described above. Standard errors in spheroid diameter were determined from three independent biological replicates.

Cylindroid Culture

Tumor cylindroids were formed by constraining spheroids between the bottom surface of a 96-well plate and the top surface of a set of polycarbonate cylindrical plugs attached to a polycarbonate lid (Fig. 2; Kasinskas and Forbes, 2006). The geometry of cylindroids forces nutrients in the bulk medium to interact only with cells at the outer edge. In this way cylindroids are similar to spheroids and are models of the microenvironments present in tumors. Tumor spheroids have been previously shown to contain proliferating cells at the periphery, necrotic cells in the core and quiescent cells between (Sutherland, 1988; Sutherland and Durand, 1984). However, unlike spheroids, cylindroids have optically accessible cores and allow for real-time quantification of the tumor microenvironment.

The gap between the bottom of the plates and the plugs was set to 150 ± 5 μm using stainless steel screws in the polycarbonate lid. The diameter of each cylindroid was dependent on the size of the spheroid used in its formation and ranged from 300 to 1,000 μm in diameter. Cylindroids were immersed in 100 μL of DMEM/HEPES media, which was added to each well with a syringe through holes adjacent to each plug in the polycarbonate lid.

Cell Viability in Cylindroids

The Live/Dead Cell-mediated Cytotoxicity Kit (L7010; Molecular Probes, Eugene, OR) was used to quantify cell viability in tumor cylindroids. To stain for viable cells, cylindroids were incubated for 24 h in 100 μL of complete media containing 1:250 (v/v) 3,3'-dihexadecyloxacyanine (DiOC18(3)). The staining media was removed and the cylindroids were washed with PBS. To stain for non-viable cells, cylindroids were incubated in 100 μL media containing 1:500 (v/v) propidium iodide for 20 min.

The localization of fluorescent dyes in cylindroids was quantified using an Olympus IX71 inverted microscope equipped with a 10X Plan-APO fluorescence objective and IPLab software (Scanalytics, Fairfax, VA). For large cylindroids, four images were tiled together to create a larger image centered on the cylindroid. Fluorescence intensity was measured as a function of radial position. Cylindroid radius was determined from transmitted light images. Radial profiles were generated from fluorescent images by averaging all of the pixel intensities at a series of radii from the cylindroid center ($r = 0$) to the cylindroid edge ($r = R$) using a script in ImageJ (NIH Research Services

Figure 2. The cylindroid device. An individual tumor cylindroid is formed by constraining a spheroid between the bottom of a well plate and a plug attached to the well-plate lid. Plugs are spaced 150 μm above the bottom of the plate.
Metabolism of Cells in Monolayer Culture

Both wild-type and HIF-1α-null cells were seeded onto three T25 cell culture flasks at a density of 200 cells/cm². Every 24 h, cells were stained with trypan blue and counted using a hemocytometer and the concentrations of glucose, lactate, glutamate, and glutamine were measured with a YSI 7100 biochemistry analyzer. The medium was not changed for the duration of the experiment (144 h).

Metabolite uptake and production rates for cells grown in monolayer cultures were determined from the concentration time profiles and cell growth profiles. Because cell number changed significantly during the experiment, the effect of growth was taken into account in the calculation of extracellular fluxes. For each cell line a specific growth rate, \( \mu \), was determined by fitting an exponential function to the cell growth profile. The metabolite uptake (or production) rates, \( v \), were calculated as functions of the cell number, \( N \); the culture volume, \( V \); and the rate of change of the metabolite concentration, \( C \).

\[
v = \frac{V}{N} \frac{dC}{dt} \tag{1}
\]

The number of cells at any time \( t \) is given by the specific growth rate.

\[
N = N_0 e^{\mu t} \tag{2}
\]

Integrating equation (1) from \( t_0 \) to \( t \) and incorporating Equation 2 produces a relation for the metabolite concentration as a function of time.

\[
C - C_0 = \frac{vN_0}{\mu V} (e^{\mu t} - e^{\mu t_0}) \tag{3}
\]

The specific uptake rate, \( v \), was determined from Equation 3 as the slope of \((C-C_0)\) versus \( N_0/\mu V(e^{\mu t} - e^{\mu t_0})\). Final uptake rates were determined by averaging the uptake rates measured for the three individual flasks. Standard errors were determined from these three independent biological replicate measurements.

Metabolism of Spheroid Cultures

Small and large spheroids were prepared for metabolic analysis in culture flasks and transferred to spinner flasks to achieve high cell numbers. Poly (2-hydroxyethyl methacrylate)-coated T225 flasks were inoculated with wild-type and HIF-1α-null cells at a density of \( 1.5 \times 10^5 \) cells/mL. Spheroids were grown for 4 days and transferred to spinner flasks containing 1 L of medium and were stirred at 100 rpm. To produce a constant cell density at the end of the experiments, 13 and 5 T225 flasks were used per spinner flask to grow small and large spheroids, respectively. Every 2 days, 700 mL of medium was replaced and 4 mL of medium was sampled to monitor cell density and spheroid size. Small and large spheroids grew to the desired sizes in approximately 6 and 15 days, respectively.

Metabolic analysis of spheroid cultures was performed in spinner flasks using isotope-enriched medium. One spinner flask was used for each of three different conditions: small wild-type spheroids, large wild-type spheroids, and large HIF-1α-null spheroids. Once the spheroids had grown to the desired size, the medium in the cultures was replaced with DMEM containing 4.5 g/L \(^{13}\)C glucose (Cambridge Isotope Laboratories). The stirrer was turned off, the spheroids were allowed to precipitate, 950 mL of old medium was removed, and 500 mL isotope-enriched medium was added. After replacing the medium, the total cell number and the concentrations of glucose, lactate, glutamine, and glutamate were measured every 6 h for 24 h.

After 24 h, intracellular \(^{13}\)C-containing metabolites were isolated from the cultures using perchloric acid (Damadian, 1981; Degani et al., 1991; Metz and Dunphy, 1996; Portais et al., 1993; Forbes et al., 2006). The medium was removed from the cultures by centrifugation, the cells were washed with 10 mL PBS, and 10 mL of ice-cold 5% perchloric acid was added. The cell lysate was neutralized with 2.5 mL 2 M KOH and was centrifuged for 5 min at 9.7 g to remove precipitated potassium perchlorate. The supernatant was frozen at \(-80^\circ\text{C}\) for 24 h and lyophilized (Labconco) overnight at \(60 \times 10^{-3}\) mBar.

Extracellular fluxes were calculated from linear fits to the metabolite concentration time profiles of glucose, glutamine, glutamate, and lactate. The slopes of the fitted functions (mmol/h/L) were divided by the average number of cells in the spinner flasks and multiplying by the volume of medium (500 mL). The average number of cells was used because the number of cells remained constant through the duration of the experiments (24 h; data not shown). Errors in the extracellular fluxes were determined from the errors in the slopes of the fitted functions and errors in the cell number measurements.

Acquisition of NMR Spectra

Lyophilized cell extracts were dissolved in 250 mL D₂O containing 0.5% sodium azide and 5 mM ethylene glycol, an NMR standard that produces a single carbon peak (Forbes et al., 2006). Samples were loaded into Shigemi NMR tubes (Shigemi, Inc., BMS-005), which double spectrometer sensitivity. Spectra were acquired with a 400 MHz Bruker Avance spectrometer at 24°C. Each spectrum was acquired with an 11 s inter-pulse delay, a 6.5 µs pulse length, and WALTZ-16 decoupling. The spectra were each an average of 4,500 scans. The \(^{13}\)C nuclear Overhauser effect was not
Corrected for; it was found to have minimal effect on the carbon peaks of interest (glutamate-2, -3, and -4 and lactate-2 and -3) relative to the ethylene glycol standard (data not shown).

**Calculation of Isotopomer Fractions**

Multiply labeled species were observed in the spectra of the cellular extracts as satellite doublet peaks surrounding the peak of the central, singly labeled species (Forbes et al., 2006). The areas of these peaks were used to determine isotopomer fractions for the observed metabolites. An isotopomer is a metabolite with one specific isotope-labeling pattern. An isotopomer fraction is the ratio of the concentration of the isotopomer to the concentration of the corresponding metabolite in the extract. The measured isotopomer fractions include contributions from all isotopomers labeled two or more bonds away from the central nucleus because NMR cannot detect $^{13}$C–$^{13}$C interactions over more than one bond length.

The concentrations of eight isotopomer groups were determined by deconvoluting the peaks with Lorenzian functions and comparing their areas to the area of the ethylene glycol standard. The total concentration of labeled glutamate and lactate in the extracts was determined using the biochemistry analyzer as described above. Isotopomer fractions were determined by dividing the isotopomer concentrations by the total concentration of the corresponding metabolite. Errors were determined from the measurements of three independent NMR spectra.

**Metabolic Pathway Model**

The measured data were converted into enzymatic fluxes using a model of the major metabolic pathways in mammalian cells (Fig. 3). This pathway model was generated from a comprehensive list of pathways by eliminating the pathways that do not significantly affect carbon flux while retaining pathways that significantly contribute to energy metabolism, biosynthesis, and isotope rearrangement (Forbes et al., 2000). This model incorporates glycolysis ($f_1$–$f_6$), the TCA cycle ($f_{13}$–$f_{17}$), the pentose phosphate pathway ($f_3$, $f_{26}$, and $f_{27}$), the malate-aspartate shuttle ($f_9$), pyruvate carboxylase ($f_{18}$), and transfer of metabolite across the mitochondrial membrane ($f_8$, $f_9$, $f_{12}$, and $f_{24}$). Two separate fluxes from α-ketoglutarate to malate ($f_{17}$ and $f_{18}$) were included in the pathway model to account for enzymatic channeling of labeled substrates by mitochondrial enzymes. The reversibility of the malate-aspartate shuttle and glutamate transport were accounted for by two association factors, $a_1$ and $a_2$, respectively.

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The pathway map (Fig. 3) was converted into a stoichiometric matrix that contained 29 fluxes and 14 intracellular metabolite balances. To render this matrix square, three sections were added: the measured extracellular fluxes, a set of independent fluxes, and a set of constraints (Forbes et al., 2001; Forbes et al., 2006). To solve the flux balance, the following assumptions were made: (1) the biosynthetic fluxes ($f_{20}$ and $f_{28}$) were constrained to be equal to their complementary biodegradation fluxes ($f_{21}$ and $f_{29}$); (2) the consumption of fatty acid ($f_{23}$) was assumed to be negligible; (3) the ratio of aspartate ($f_{28}$) to glutamate ($f_{20}$) incorporation into protein was assumed to be equal to their relative proportion in mammalian cell proteins; and (4) the flux of citrate out of the mitochondria ($f_{12}$) was constrained to zero because its value cannot be distinguished from the net flux of malate flux ($f_9$) by isotope tracing (Forbes et al., 2000, 2006).

**Computational Analysis**

The algorithm used to determine intracellular fluxes contains two distinct components: isotopomer path tracing (Forbes et al., 2001) and simulated annealing (Schmidt et al., 1999). Isotopomer path tracing was used to relate intracellular fluxes to the isotope-labeling pattern by tracing...
carbon atoms through the metabolic pathway map. The tracing algorithm generated a series of algebraic equations from the stoichiometric matrix and the atom-mapping matrices, which were used to generate the values in the isotope-labeling matrix (Forbes et al., 2001).

Simulated annealing was used to determine the values of the intracellular fluxes that best fit the measured isotope-labeling pattern (Forbes et al., 2006). The algorithm was initiated with estimated values for the independent fluxes. It then: (1) used the isotope-labeling matrix to determine the isotopomer pattern that would result from these guessed flux values; (2) compared the pattern to the measured data; and (3) determined the resulting least-squares error. The independent fluxes were adjusted until this error was minimized. An annealing constant of 1,000 with an initial temperature of 10 was used, as described by Schmidt et al. (1999). To approach a global minimum in a reasonable time, each simulation of 10,000 iterations was repeated 10 times with the results of the previous simulation as the initial guesses for the next iteration.

Error Analysis

Two types of data were used to calculate intracellular fluxes: extracellular fluxes and the isotopomer fractions. Errors in these two data types were determined from the errors of multiple individual measurements. Errors in the extracellular fluxes were determined from uncertainty in the slope of the metabolite concentration profiles and uncertainty in the average cell number. Errors in the isotopomer fractions were determined from uncertainty in labeled metabolite peak area and uncertainty in the total metabolite concentration.

Monte Carlo simulations were used to determine the errors in the intracellular fluxes. As described above, errors were measured for all of the input data, including the extracellular fluxes and the isotopomer fractions. All of these measured errors were assumed to be random and uncorrelated. The normal distribution functions, centered on the measured averages with confidence intervals equal to the measured errors, were used as the input to the simulation. Monte Carlo error estimation was performed as follows: (1) a set of inputs was randomly generated from probability density functions; (2) fluxes were calculated from the set of inputs using the flux analysis algorithm; (3) the process of generating inputs and running the algorithm was iterated 100 times generating 100 independent flux results; and (4) the error in the intracellular fluxes was determined from the standard deviation of the set of flux results. Statistical significance for all measurements and results was determined using Student’s t-test.

Figure 4. Spheroid growth dynamics. A: Transmitted image of wild-type spheroids. B, C: The average diameters of 4-day-old (B) and 9-day-old (C) spheroids, grown in spinner flasks, were 197 ± 82 and 597 ± 135 μm, respectively. Frequency indicates the fraction of cells within each size range. D: Growth of spheroids composed of wild-type (HIF-1α+/+) and null (HIF-1α−/−) cells in methacylate-coated flasks. Error bars represent the standard error of the mean from triplicate experiments.
Results

Spheroid Growth

The size distribution of spheroids in spinner flasks (Fig. 4A) was controlled by the length of time they were grown in culture. The average sizes of spheroids grown for 4 and 9 days were 197 ± 82 and 597 ± 135 μm, respectively (Fig. 4B,C). In methacrylate-coated flasks spheroids of both wild-type and HIF-1α-null cells grew linearly and there was no discernable difference in their growth rates (Fig. 4D).

Cell Viability in Cylindroids

Cylindroids of different sizes and composed of wild-type and HIF-1α-null cells were stained to determine how cell viability is affected by microenvironment and cell type (Fig. 5A–H). Metabolic microenvironments arise in cylindroids because of nutrient diffusion gradients (Sutherland, 1988), which are dependent on cylindroids size and the distance of cellular regions to nutrients supplied in the bulk. In Figure 5, live and dead cells are stained green and red, respectively. The stain for dead cells, propidium iodide, identifies cells that have permeable cell membranes and includes cells that have died by either apoptosis or necrotic lysis. In the intensity profiles, which represent cell viability as a function of radial position, the number of viable cells (green) decreased and the number of dead cells (red) increased towards the center of the cylindroids (Fig. 5I–L). No viable cells were observed the centers of the cylindroids (data not shown). The increase in green fluorescence intensity in the cylindroid centers was due to autofluorescence of the necrotic tissue. Unstained cylindroids had green fluorescent central regions similar to the inner region (<150 μm) of the radial fluorescence profiles in Figure 5I–L (data not shown). The region between the outer edge of the cylindroid and the minimum in the green fluorescence intensity profile was defined as the viable rim. Based on the radial intensity profiles of 36 cylindroids, the thickness of the viable rim was found to be independent of cell type (P < 0.05) and cylindroid size (P < 0.05; Fig. 6). Across all observed cylindroids the viable rim was 197 ± 5 μm thick. Spheroids smaller than 400 μm in diameter did not contain necrotic cells (data not shown).

Cell Metabolism in Monolayer Culture

When grown in monolayer culture the specific growth rates of HIF-1α-null cells, 0.040 ± 0.004/h, was not significantly different from wild-type cells, 0.043 ± 0.003/h (Fig. 7A). These growth profiles are similar to those obtained by Seagroves et al. (2001), who reported that wild-type and HIF-1α-null cells do not have different growth rates under normoxic conditions. Similarly, the concentration profiles of glucose and lactate (Fig. 7B) and glutamine and glutamate (Fig. 7C), which were used to calculate extracellular flux, were not different for the two cell lines. The high rates of glutaminolysis in Figure 7C are typical of cancerous cells and are believed to enable increased biosynthesis (Forbes et al., 2006; Mazurek et al., 1999; Medina, 2001).
Cell Metabolism in Spheroids

The metabolism and growth of three different spheroid conditions were determined: small wild-type spheroids, large wild-type spheroids, and large HIF-1α-null spheroids. Comparison of small to large wild-type spheroids was used to determine the metabolism of quiescent cells, and comparison of wild-type to HIF-1α-null large spheroids was used to determine the effects of HIF-1α.

The final number of cells and the diameter of the spheroids were both significantly ($P < 0.05$) less in small compared to large spheroid cultures (Fig. 8A,B). This difference in size was created by growing spheroids longer in culture: 6 and 15 days for small and large spheroids, respectively. The average diameters of the small wild-type, large wild-type, and large HIF-1α-null spheroids were 275, 809, and 839 μm, respectively (Fig. 8B). For all three conditions, cell density did not change significantly during the 24 h course of experiments from isotope-enriched medium addition to metabolite extraction (data not shown).

The concentrations of four cellular metabolites (glucose, lactate, glutamine, and glutamate) were measured as a function of time to determine extracellular fluxes. The concentration profiles were shallower for small spheroids (Fig. 8C,D) than for large spheroids (Fig. 8E,F) because there are fewer cells in the flasks, a difference that was compensated for in the extracellular flux calculation. The concentration profiles of glucose and lactate (Fig. 8E) and glutamine and glutamate (Fig. 8F) were almost identical for wild-type and HIF-1α-null spheroids.

**Figure 6.** The thickness of the viable rim is independent of both spheroid size and cell type. Error bars represent standard errors. At least four independent measurements were made for each group of cylindroids.

**Figure 7.** Monolayer growth of wild-type and HIF-1α-null cells. A: Cell growth profiles for wild-type, •, and HIF-1α-null, ○, cells. B: Glucose, □ & △, and lactate, ▲ & ▲, concentration profiles, for wild-type (filled symbols) and HIF-1α-null (hollow symbol) cells. C: Glutamine, ● & ○, and glutamate, ◆ & •, concentration profiles. All data points represent the average of five replicate flasks. Error bars represent standard errors.
Extracellular Metabolite Fluxes

Extracellular fluxes (Fig. 9) were calculated by fitting linear functions to the metabolite concentration profiles (Fig. 8) and dividing the slope by the number of cells in the cultures (Fig. 8A). The metabolic model in Figure 3 specifies the directions of the extracellular fluxes, which are all reported as positive values. In both monolayer and spheroid cultures no statistical difference in the extracellular flux of any metabolite was observed between wild-type and HIF-1α-null cells. However, spheroid size affected the extracellular fluxes: small spheroids had significantly (P < 0.05) larger uptake of glucose and production of lactate and glutamate (Fig. 9B,D). There was no significant difference between the extracellular fluxes measured in small spheroids and cells grown in monolayer. The extracellular fluxes of glucose (P < 0.05), lactate (P < 0.01), glutamate (P < 0.01), and glutamine (P < 0.01) were all significantly less in large spheroids compared to monolayer culture.

Isotope Distribution

Three 13C-NMR spectra were acquired (Fig. 10) and eight isotopomer fractions (Table I) were determined for each of the three spheroid cultures. The notation of Table I includes both singly and doubly labeled metabolites. For example, lactate-2 and lactate-23 indicate lactate labeled only on 2nd carbon and at both the 2nd and 3rd carbons, respectively. The amount of isotope incorporated into lactate-3 was larger than the other metabolite carbons because of direct production via glycolysis. Glutamate-4 is greater than glutamate-2 and -3 as a result of dilution in the TCA cycle. Similar to extracellular fluxes, no significant difference was...
observed between the isotopomer fractions of the two large wild-type and HIF-1α-null spheroid cultures. All isotopomer fractions had greater values for large spheroids compared to small spheroids, and glutamate-2 (P < 0.05) and glutamate-23 (P < 0.01) were significantly greater in large spheroids.

**Intracellular Fluxes**

The intracellular fluxes calculated by the flux analysis algorithm from the extracellular fluxes and isotopomer fractions are presented in Table II. The reversibility of the malate-aspartate and glutamate fluxes are presented in Table III. A selection of important fluxes is highlighted in Figures 11 and 12. All fluxes were greater in small compared to large spheroids including both energetic and biosynthetic fluxes. Small spheroids had significantly greater glycolytic (f1, P < 0.01), lactate producing (f10, P < 0.01) and TCA cycle (f13, P < 0.05) fluxes (Fig. 11). In addition, the major biosynthetic fluxes in the pathway model, including the pentose phosphate pathway (f5, P < 0.05), and pyruvate carboxylase (f18, P < 0.01), were significantly greater in small spheroids. There was no significant difference between the fluxes of wild-type and HIF-1α-null spheroids (Fig. 12), with the exception of the TCA cycle fluxes (f8, f11, f13, f15, and f16), which were slightly larger (P < 0.05) in wild-type spheroids (Table II). The rates of glycolysis and lactate production were unchanged between the two spheroid types.

The ratio of anaerobic to aerobic metabolism (defined as the production of lactate, f10, divided by the TCA cycle flux, f13) was 4.8 ± 0.9, 6.5 ± 0.9, and 6.1 ± 0.5 for small wild-type, large wild-type, and large HIF-1α-null spheroids, respectively. In all spheroids, intra-mitochondrial malic enzyme (f22) was very small, indicating that anaplerotic carbons were not directly oxidized within mitochondria (Table II). No significant difference was observed in the rate of glutaminolysis between any spheroid types.

**Discussion**

**Metabolism of Quiescent Cells in Spheroids**

The comparison of small and large spheroids is a good model of quiescent cells. The two different sizes of spheroids have considerably different ratios of proliferating...
to quiescent cells (Fig. 13). The volume fractions in Figure 13 are reported to illustrate this composition difference and were not used in metabolic flux calculations. Using the cylindroid model, the thickness of the viable region was determined to be approximately 200 μm, regardless of cell type or spheroid size (Fig. 6). The viable rim thickness is similar in spheroids and cylindroids because of their similar geometry and similar nutrient diffusion gradients. In both cultures, nutrients diffuse from the bulk into the center of the tissue where cells die because of nutrient depletion. Sutherland (1988) reported that the thickness of the proliferating region in spheroids is typically three to five cell layers, or 75 μm. By subtracting, the thickness of the quiescent region is approximately 125 μm. Based on these values the volume ratios of quiescent to proliferating cells for large (800 μm) and small (275 μm) spheroids were 89% and 10%, respectively (Fig. 13). Necrotic cells are not metabolically active and would not affect metabolic flux measurements. Because small spheroids are almost entirely composed of proliferating cells, metabolic fluxes measured in small spheroids are representative of the metabolism of proliferating cells. Large spheroids, on the other hand, are composed of nearly equal volumes of proliferating and quiescent cells and metabolic fluxes measured in large spheroids are representative of the metabolism of both proliferating and quiescent cells.

### Table 1. Isotopomer fractions of lactate and glutamate.

<table>
<thead>
<tr>
<th></th>
<th>Small wild-type</th>
<th>Lactate 2</th>
<th>Lactate 3</th>
<th>Lactate 23</th>
<th>Glutamate 2</th>
<th>Glutamate 23</th>
<th>Glutamate 3</th>
<th>Glutamate 4</th>
<th>Glutamate 43</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate</td>
<td></td>
<td>7.0 ± 0.7</td>
<td>10.1 ± 1.4</td>
<td>11.6 ± 1.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactate 3</td>
<td></td>
<td>433 ± 43</td>
<td>556 ± 78</td>
<td>588 ± 60</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactate 23</td>
<td></td>
<td>3.8 ± 0.4</td>
<td>6.0 ± 0.9</td>
<td>6.5 ± 0.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamate 2</td>
<td></td>
<td>28.5 ± 2.8</td>
<td>55.9 ± 7.7</td>
<td>60.4 ± 6.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamate 23</td>
<td></td>
<td>2.4 ± 0.2</td>
<td>19.3 ± 2.7</td>
<td>18.9 ± 2.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamate 3</td>
<td></td>
<td>23.9 ± 2.4</td>
<td>35.1 ± 4.7</td>
<td>23.6 ± 2.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamate 4</td>
<td></td>
<td>113 ± 11</td>
<td>221 ± 56</td>
<td>210 ± 22</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamate 43</td>
<td></td>
<td>15.5 ± 1.6</td>
<td>42.1 ± 10.6</td>
<td>40.8 ± 4.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*All values have been multiplied by 1,000. Errors were calculated based on three independent spectra for each cell line and spheroid size. Significance is indicated for each isotopomer fraction based on Student’s t-test comparison between small and large spheroids. There was no significant difference between the isotopomer fractions of wild-type and HIF-1α-null spheroids.

*P < 0.05.

**P < 0.01.
fluxes measured in large spheroids are representative of an average of both cell types. Although the metabolism measured in large spheroids is not equivalent to the metabolism of quiescent cells, the difference between the metabolism of small and large spheroids gives an indication of how metabolism changes in cells as they become quiescent.

Large spheroids, and hence quiescent cells, had lower measured rates of energy metabolism and biosynthesis (Fig. 11). This was observed as reduced extracellular fluxes (Fig. 9) and calculated intracellular fluxes (Table II). The ratio of anaerobic to aerobic metabolism was also greater in (Fig. 11). This was observed as reduced extracellular fluxes (Fig. 9) and calculated intracellular fluxes (Table II). The ratio of anaerobic to aerobic metabolism was also greater in large spheroids, indicating that quiescent cells in the center of large spheroids have less access to oxygen and must rely on glycolysis for energy. From these results it can be interpreted that quiescent cells uptake less nutrients because of diffusion resistance through the outer layers of the spheroids. The cells therefore have less energy for regular cellular processes and growth.

Two important biosynthetic fluxes, the pentose phosphate pathway (f12) and pyruvate carboxylase (f13), were significantly reduced in large spheroids (Fig. 11). The pentose phosphate pathway generates ribose 5-phosphate and NADPH, which are necessary for the formation of nucleotides and lipids (Forbes et al., 2000). Pyruvate carboxylase is the primary anaplerotic reaction in cells. It increases the pools of TCA cycle metabolites, which are necessary precursors for amino acids, nucleotides, and lipids (Forbes et al., 2000). Quiescent cells in spheroids are therefore producing these precursors at a slower rate than proliferating cells. Indeed, Sutherland, Feyer, and coworkers determined previously using tritiated thymidine that quiescent cells dissociated from spheroids do not actively synthesize DNA (Freyer and Sutherland, 1980; LaRue et al., 2004).

**Table II.** Intracellular fluxes.

<table>
<thead>
<tr>
<th>Enzyme/Pathway</th>
<th>Small wild-type</th>
<th>Large wild-type</th>
<th>Large HIF-1α-null</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexokinase/glucokinase</td>
<td>f1</td>
<td>503 ± 39</td>
<td>272 ± 31**</td>
</tr>
<tr>
<td>Phosphoglucone Isomerase</td>
<td>f2</td>
<td>493 ± 38</td>
<td>272 ± 31**</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>f3</td>
<td>10 ± 4</td>
<td>0.30 ± 0.10*</td>
</tr>
<tr>
<td>Phosphofructokinase</td>
<td>f4</td>
<td>499 ± 39</td>
<td>272 ± 31**</td>
</tr>
<tr>
<td>Aldolase</td>
<td>f5</td>
<td>499 ± 39</td>
<td>272 ± 31**</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>f6</td>
<td>1002 ± 77</td>
<td>544 ± 61**</td>
</tr>
<tr>
<td>Glutamate production</td>
<td>f7</td>
<td>25 ± 1</td>
<td>7.6 ± 0.3**</td>
</tr>
<tr>
<td>Pyruvate transport into mitochondria</td>
<td>f8</td>
<td>188 ± 29</td>
<td>77 ± 6*</td>
</tr>
<tr>
<td>Malate aspartate shuttle</td>
<td>f9</td>
<td>82 ± 24</td>
<td>30 ± 3</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>f10</td>
<td>896 ± 84</td>
<td>497 ± 59**</td>
</tr>
<tr>
<td>Pyruvate dehydrogenase</td>
<td>f11</td>
<td>138 ± 22</td>
<td>68 ± 3*</td>
</tr>
<tr>
<td>Citrate transport to cytoplasm</td>
<td>f12</td>
<td>Set to zero</td>
<td></td>
</tr>
<tr>
<td>Citrate synthase</td>
<td>f13</td>
<td>138 ± 22</td>
<td>68 ± 3*</td>
</tr>
<tr>
<td>Glutaminase</td>
<td>f14</td>
<td>57 ± 25</td>
<td>29 ± 7</td>
</tr>
<tr>
<td>Isocitrate dehydrogenase</td>
<td>f15</td>
<td>138 ± 22</td>
<td>68 ± 3*</td>
</tr>
<tr>
<td>Enzymatic channeling in TCA cycle I</td>
<td>f16</td>
<td>160 ± 34</td>
<td>72 ± 8*</td>
</tr>
<tr>
<td>Enzymatic channeling in TCA cycle II</td>
<td>f17</td>
<td>9 ± 7</td>
<td>18 ± 14</td>
</tr>
<tr>
<td>Pyruvate carboxylase</td>
<td>f18</td>
<td>51 ± 8</td>
<td>9 ± 5**</td>
</tr>
<tr>
<td>Malic enzyme (extra-mitochondrial)</td>
<td>f19</td>
<td>82 ± 24</td>
<td>30 ± 3</td>
</tr>
<tr>
<td>Glutamate incorporation into protein</td>
<td>f20</td>
<td>115 ± 34</td>
<td>0.30 ± 0.02*</td>
</tr>
<tr>
<td>Glutamate production from protein</td>
<td>f21</td>
<td>115 ± 34</td>
<td>0.30 ± 0.02*</td>
</tr>
<tr>
<td>Malic enzyme (intra-mitochondrial)</td>
<td>f22</td>
<td>0.6 ± 0.3</td>
<td>0.30 ± 0.04</td>
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<tr>
<td>Fatty acid consumption</td>
<td>f23</td>
<td>Set to zero</td>
<td></td>
</tr>
<tr>
<td>Aminotransferases (Ala and Asp)</td>
<td>f24</td>
<td>32 ± 25</td>
<td>21 ± 7</td>
</tr>
<tr>
<td>Triose phosphate isomerase</td>
<td>f25</td>
<td>499 ± 39</td>
<td>272 ± 31**</td>
</tr>
<tr>
<td>Transaldolase</td>
<td>f26</td>
<td>7 ± 2</td>
<td>0.21 ± 0.06*</td>
</tr>
<tr>
<td>Transketolase</td>
<td>f27</td>
<td>3 ± 1</td>
<td>0.09 ± 0.03*</td>
</tr>
<tr>
<td>Aspartate incorporation into protein</td>
<td>f28</td>
<td>102 ± 30</td>
<td>0.27 ± 0.02*</td>
</tr>
<tr>
<td>Aspartate production from protein</td>
<td>f29</td>
<td>102 ± 30</td>
<td>0.27 ± 0.02*</td>
</tr>
</tbody>
</table>

*Values are reported in units of μmol/h/10^9 cell. Significance is indicated for each flux based on Student’s t-test comparison between: (1) small and large wild-type spheroids; and (2) large wild-type and HIF-1α-null spheroids. Two fluxes (f12 and f23) were set to zero in the flux determination algorithm and were not calculated.

**Table III.** Transmembrane exchange fluxes.

<table>
<thead>
<tr>
<th>Flux</th>
<th>Small wild-type</th>
<th>Large wild-type</th>
<th>Large HIF-1α-null</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malate-aspartate shuttle</td>
<td>α1</td>
<td>0.78 ± 0.21</td>
<td>0.005 ± 0.14*</td>
</tr>
<tr>
<td>Glutamate transport</td>
<td>α2</td>
<td>0.46 ± 0.10</td>
<td>0.55 ± 0.07</td>
</tr>
</tbody>
</table>

*The extent of reversibility is reported as normalized association factors, which scale from irreversible, 0, to rapidly reversible, 1. Significance is based on Student’s t-test between small and large wild-type spheroids.

**P < 0.05.**
HIF-1α has Minimal Affect on Cell Survival and Metabolism in Three-Dimensional Tissue

In our experiments, the absence of HIF-1α did not affect any of our results, including: (1) cell survival in cylindroids; (2) the uptake and production of nutrients in monolayer and spheroids; (3) isotope labeling of spheroids; and (4) the intracellular metabolism of cells in spheroids. From these results and our results with small and large spheroids we conclude that HIF-1α is not able to regulate the metabolism of cancer cells in the presence of the other gradients that are found in three-dimensional tumor tissue.

By quantifying viability in different-sized cylindroids we have shown that the viable rim thickness does not change with cylindroid size (Figs. 5 and 6), which suggests that diffusible factors control cell survival in spheroids, which include oxygen, glucose, other nutrients and protein growth factors. These factors can be divided into oxygen-dependent and oxygen-independent mechanisms. In addition, the oxygen-dependent mechanisms can be further divided into those controlled by HIF-1α and those independent of HIF-1α. For example, the reduction of ATP production in mitochondria in the absence of environmental oxygen is a HIF-1α-independent mechanism.

Oxygen and glucose gradients in spheroids were studied previously by Sutherland and coworkers, who showed that spheroids contain gradients of both oxygen and glucose and that both of these factors interdependently cause cell death (Freyer and Sutherland, 1986; Sutherland, 1986). Our observation that the anaerobic to aerobic ratio was greater in larger compared to smaller spheroids suggests that the oxygen gradient present in spheroids does affect cellular metabolism. However, this ratio was unchanged between wild-type and HIF-1α-null spheroids suggesting that the response to oxygen gradients in spheroids is independent of HIF-1α.

Figure 11. Intracellular metabolism of small and large spheroids. Upper and lower values are fluxes from small, S, and large, L, wild-type spheroids, respectively. Fluxes have units of \( \mu \text{mol/h/}10^9 \text{cell} \). Significance is indicated based on Student’s \( t \)-test (\( t^* \), \( P < 0.05 \); \( ^* \), \( P < 0.01 \)).
Most of the metabolic effects of HIF-1α require available glucose and a source of cellular energy (Fig. 1). The glycolytic flux was significantly less in large spheroids, which contain quiescent cells, because of the lack of available glucose in the spheroid center (Table II, Fig. 11). However, there was little difference between the glycolytic fluxes between wild-type and HIF-1α-null spheroids (Table II, Fig. 12). Seagroves et al. (2001) showed previously that HIF-1α enables cells to survive in hypoxic environments if they are well supplied with glucose. However, in environments that are hypoxic and low in glucose HIF-1α does not provide any survival advantage (Seagroves et al., 2001). These observations suggest that the glucose gradients present in spheroids limit the effects of HIF-1α.

In addition, the absence of HIF-1α did not affect the rates of lactate production and glycolysis in spheroids (Fig. 12), despite its known effect on the expression of most enzymes in glycolysis. In monolayer cultures HIF-1α-null cells have decreased lactate production compared to wild-type cells under hypoxic conditions (Seagroves et al., 2001). If cells in three-dimensional tissues responded to oxygen gradients primarily through HIF-1α-dependent mechanisms, then HIF-1α-null spheroids would have considerably lower rates of lactate production compared to wild-type spheroids. Although the availability of oxygen for respiration appears to affect the metabolism of the cells in spheroids, our results suggest that this is not mediated by HIF-1α.

This result, that HIF-1α does not regulate cellular metabolism in spheroids, suggests that the microenvironment of tumors should be carefully taken into account when designing HIF-1α-targeted therapies. Hypoxic regions in mouse tumors have been shown to often also be low in

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**Figure 12.** Intracellular metabolism of wild-type and HIF-1α-null spheroids. Upper and lower values are fluxes from wild-type, Wt, and HIF-1α-null, N, large spheroids, respectively. Fluxes have units of μmol/h/10^9 cell. Significance is indicated based on Student’s t-test (*, P < 0.05).
glucose (Walenta et al., 2001). In tumors where regions of hypoxia are co-localized with regions of low glucose, HIF-1α would have little effect on cell survival and metabolism. From our results, the role of HIF-1α appears to enable cells to utilize available nutrients when they are in low-oxygen, nutrient-rich environments. Therefore, to determine the effectiveness of HIF-1α-based therapies, it will be important to determine the extent that hypoxia in human tumors is correlated with low-glucose environments.

Conclusions

Using metabolic flux analysis we determined that there was little difference between the metabolism of wild-type and HIF-1α-null cells grown in spheroids. Comparatively, we have shown that quiescent cells in spheroids have lower biosynthetic and aerobic respiration rates by comparing the metabolic microenvironment should be taken into account when developing therapies that target HIF-1α because it may have less function in three-dimensional culture affects cellular metabolism. They also suggest that the metabolic microenvironment should be taken into account when developing therapies that target HIF-1α because it may have less function in three-dimensional tissues compared to monolayer culture.

We would like to thank the NIH (grant # R21 CA112335-01A1) for financial support of this project.

References


Kim and Forbes: Flux Analysis of HIF-1α Spheroids


Mazurek S, Eigenbrodt E, Failing K, Steinberg P. 1999. Alterations in the pentose phosphate pathway and pyruvate carboxylase. The primary biosynthetic pathways that were less active were the pentose phosphate pathway and pyruvate carboxylase. These results show how diffusion of metabolites in three-dimensional culture can affect cellular metabolism. They also suggest that the metabolic microenvironment should be taken into account when developing therapies that target HIF-1α because it may have less function in three-dimensional tissues compared to monolayer culture.

We would like to thank the NIH (grant # R21 CA112335-01A1) for financial support of this project.

References


