ANTICANCER RESEARCH *26*: 4155-4162 (2006)

The Proliferative Response of HeLa Cells to 2-Deoxy-D-Glucose Under Hypoxic or Anoxic Conditions: An Analogue for Studying some Properties of *In Vivo* Solid Cancers

K.M. ANDERSON[1], P. TSUI[1,2], P. GUINAN[1,2] and M. RUBENSTEIN[1,2]

[1]*Hektoen Medical Research Institute and* [2]*The Cook County Hospital, Chicago, IL 60612, U.S.A.*

Abstract. *Background: Hypoxic cancer cells located beyond the diffusion path of sufficient oxygen are considered a nidus of therapeutic failure. Due to the dependence of many malignantly transformed cells on glycolysis for metabolic energy, inhibiting this and other sources of energy should seriously reduce cell viability and proliferation, additively or even synergistically. Materials and Methods: To try and duplicate in vitro some of the features of in vivo cancer cells likely to resist therapy, HeLa cells were incubated with sub-lethal concentrations of 2-deoxy-D-glucose under aerobic, hypoxic or virtually anoxic conditions, verified by increased synthesis of Hif-1α, and their replication and survival determined. MK 886, an inhibitor of mitochondrial function was used to estimate participation of that organelle in energy metabolism. Results: Culture of cervical cancer-derived HeLa cells with 2-deoxy-D-glucose under these restrictive conditions did not reduce their proliferation or viability to the expected extent. Their surprisingly robust survival included the anticipated increase in dependence upon glycolysis and implied a likely entrainment of other constitutive and possibly facultative energy sources and pathways. Increased synthesis of Hif-1α, increased binding to its consensus sequence and reduced inhibition from MK 886 in cells under oxygen-deficient environments confirmed the presence of restrictive conditions. Conclusion: Efforts to suppress HeLa cell survival by reducing glucose consumption and metabolic energy from ambient oxygen may require inhibition of multiple energy sources, possibly not all of them identified. In vitro assessment of agents directed against sources of metabolic energy or of other therapeutic agents against these or additional potential targets should include studies under hypoxia and relative anoxia. In this way, the possible responses of in vivo hypoxic or anoxic cancer cells believed to contribute to therapeutic failure may be estimated.*

Correspondence to: Dr. K.M. Anderson, Hektoen Medical Research Institute LLC, 627 S. Wood. Street, Chicago, IL 60612, U.S.A. Tel: 312-864-0564, Fax: 312-864-9569, e-mail: Kanderso427@aol.com

Key Words: 2-Deoxy-D-glucose, hypoxia, HeLa, anoxia, glycolysis.

At first view, dual suppression of cancer cell aerobic and anaerobic glucose-dependent metabolic energy should be extremely lethal. Reducing mitochondrial or cytoplasmic, non-mitochondrial sources of compounds exhibiting high free energy of hydrolysis inhibits cell proliferation (1, 2), and augments cell death in mutants lacking mitochondrial DNA with their reduced oxidative phosphorylation and auxotrophy for uridine (2, 3). Inhibition of glucose metabolism, alone (2, 4) or combined with traditional chemotherapy (5) increases the killing of cultured cancer cells. Cells located in hypoxic environments and strongly dependent upon glycolysis (6) include clones refractory to therapy (7). Finally, hypoxic cells with augmented glycolysis resist ionizing radiation (8).

Observations by Warburg of increased cancer cell glycolysis nearly a century ago (9) and the Pasteur response to hypoxia of increased glycolysis with reduced oxidative phosphorylation (10), recently associated with defective P53 (11), the altered distribution of glycolytic enzyme activities in hypoxic cells (12), the role of factors such as the AKT serine / threonine kinases in augmenting cancer cell aerobic glycolysis (13) and increased local concentrations of lactic acid and decreased extra-cellular pH (8) all direct attention to altered energy utilization. However, it was reported that ATP synthesis in MCF-7 breast cancer cells was 80% oxidative and 20% glycolytic (14). Contributions to the oxidative component were 10% from glucose, 14% glutamine, 7% palmitate, 4% oleate and 65% from unidentified sources. Glucose utilization, including glycolysis and oxidation contributed 29% to total ATP turnover, glutamine, 11% and combined, 40%. Nearly 60% of the sources for ATP turnover could not be accounted for.

The use of cell aggregates or "spheroids" to mimic the *in vivo* behavior of cancer cells is a very useful technique (15). Oxygen penetrates several hundred microns or about 0.1-0.2 mm into

an aggregate, and depending upon its size, central regions of hypoxia are present. Aggregates can differ in size, the diffusion of agents is limited and cells may not be equally affected.

Leaving aside questions of cancer cell-targeting (1, 16), we were interested in the consequences of the combined, but individually *sub-lethal,* inhibition of oxidative and non-oxidative energy on HeLa cells. The effect of hypoxia or virtual anoxia on the anti-proliferative response to 2-deoxy-D-glucose, an inhibitor of glycolysis (2, 4, 5), was examined. It is known that in HeLa cells, glutamine also serves as an important source of metabolic energy (17). In some experiments a mitochondria-oriented inhibitory agent, MK 886, exhibiting a number of identified effects on cell function leading to cessation of cell division and induction of apoptosis and related forms of programmed cell death, was used to assess indirectly the contribution to metabolic energy from mitochondria (18-20). We intended to establish culture conditions for attached hypoxic or anoxic HeLa cells that mimicked the environment of cells putatively responsible *in vivo* for failure of therapies, initially by comparing 150 μl with 300 μl incubation volumes in 96-well plates. The growth and survival-response to 2-deoxy-D-glucose would reflect the dependence upon glycolysis for metabolic energy. We thought that the increase in the oxygen diffusion path-length of the larger volume would provide a more hypoxic environment for HeLa cells attached to the bottom of the wells. Subsequently, we used hypoxic or virtually anoxic environments to examine this question, confirming the presence of such states by the up-regulation of Hif-1α. The metabolic virtuosity of HeLa cells under very unfavorable culture conditions has been underestimated. This raises a number of questions regarding efforts to inhibit hypoxic cancer cells primarily by reducing their metabolic energy. Thus, any proposed therapy first examined *in vitro* should include studies under the restrictive conditions of hypoxia and virtual anoxia, mimicking a source of resistance to therapy and a site of putative cancer "stem" cells (21).

Materials and Methods

Cell culture. In most experiments, HeLa cells were cultured for 72 h in 96-well microtiter plates at 37˚C and 5% CO_2 ("normoxic" conditions). Other plates were cultured in narrow scintillation vials able to contain up to 5.5 ml of medium to create more hypoxic environments at the bottom of the vessel, in 96-well plates or Pasteur plates filled with medium and covers sealed with vaseline ("hypoxic") or incubated in 96-well plates or 75 cm^2 flasks in a bell jar at 37˚C under a predominant atmosphere of nitrogen ("anoxic" conditions). The RPMI-1640 medium contained GlutaMax™, 10% FBS, 50 U/ml penicillin G, 50 μg/ml streptomycin and 25 mM HEPES. Cells were sub-cultured the day before use, detached with 0.25% trypsin-0.03% EDTA and their initial number and viability determined with trypan blue and a hemocytometer. Media in 96-well plates contained RPMI diluted with PBS to 3.3% FBS and 3.3 mM D-glucose. Cell proliferation and viability was measured with an MTS or, less frequently, WST-1 assay according to instructions provided with the commercial kits (Promega, Madison, WI, USA; Roche Diagnostics, Panzberg, Germany). Two-deoxy-D-glucose was prepared at 3 mg/ml in Dulbecco's PBS to a final maximum concentration of 6 mM; MK 886 40 μM was prepared from a 120 mM stock in dimethyl sulfoxide. The DMSO vehicle at a maximum concentration of 0.1% has not altered cell numbers or their viability.

As it was necessary to demonstrate that conditions of hypoxia or of virtual anoxia were established by the means described, three procedures for demonstrating augmented Hif-1α were performed.

Histochemical-immuno-assay for Hif-1α. Hif-1α mouse monoclonal antibody NB 100-123 from Novus Biologicals (Littleton, CO, USA) was diluted 1:100 with PBS; normal mouse serum was the negative control. Cells grown under normal, vaseline-sealed or nitrogen-exposed conditions in 8-chambered slides were washed with PBS, blocked with mouse serum for 20 min, washed x 3 with PBS, incubated overnight with the primary antibody at 4˚C, washed with PBS followed by biotinylated anti-mouse antibody, HRP streptavidin complex, and horseradish peroxidase substrate/DAB chromagen added in steps separated by PBS washes according to the instructions in an "Immunocruz" staining kit from Santa Cruz (Santa Cruz, CA, USA). Images were scanned and recorded with a visible/fluorescence photomicroscope.

Western blotting of Hif-1α. Western blotting of Hif-1α was performed according to a common procedure. Cells were detached into a hypotonic lysis buffer containing N-lauroylsarcosine (0.25%) and PMSF or an anti-proteinase pellet (22). Aliquots from the 3 types of samples containing 15 μg or more of protein, depending upon the experiment, were subjected to SDS-PAGE and transferred to VPDF membranes overnight at 4˚C with 100 ma current, according to instructions accompanying the BioRad apparatus. Using a Bio-Rad immuno-blot assay kit (Bio Rad, Hercules, CA, USA), membranes washed with tris-buffered saline (TBS), blocked with 3% gelatin or BSA in TBS, washed in tris-buffer containing 0.02% tween 20 (TTBS) at RT for 2 h, probed overnight at RT with Novus anti Hif-1α antibodies 100-123 or 100-105, washed with TTBS followed by TBS to remove residual tween and incubated at RT for 2 h with the specific secondary antibody coupled to alkaline phosphatase. After washing in TBS and addition of color-reagents, immuno-reactive material was detected within 20-30 min at RT and images recorded.

Hif-1α transcription factor assay. A "TransAM™" Hif-1 transcription factor kit from Active Motif (Carlsbad, CA, USA) was employed to further confirm the results of histochemistry and Western blotting and implicate binding of Hif-1α to its consensus sequences. The kit is provided in a 96-well format with wells containing an immobilized hypoxia response-element consensus sequence, to which nuclear extract is added, followed by antibody against Hif-α and subsequently a second antibody coupled with horseradish peroxidase. After incubation according to the instructions in the kit, optical density was read with a plate reader at 450 / 650 nm. HeLa cell nuclear extract was obtained using an Active Motif nuclear extraction kit which includes cell lysis in hypotonic buffer containing phosphatase inhibitors, addition of Nonident P-40 and differential extraction and centrifugation to separate the cytoplasmic from the nuclear fraction. Details of these procedures were performed according to the instructions provided.

Results

We were interested in mimicking *in vitro* the regional hypoxic environment of *in vivo* solid cancers, initially by comparing the effect of increasing the path length of diffusion of oxygen in 96-well microtiter plates. Cell proliferation was measured in wells containing 150 and 300 μl volumes with constant concentrations of 2-DG and glucose bathing HeLa cell monolayers resulting from 10,000 of cells in 150 μl or 20,000 cells in 300 μl. Initially, we compared cells in these volumes incubated under 5% CO_2 ("aerobic conditions"), representing, respectively, 0.45 and 0.91 cm oxygen diffusion paths through 0.33 cm^2 cross-sectional areas.

Using 10 or 20 thousand cells in 150 or 300 μl, respectively, under aerobic conditions, effectively reduced cell proliferation at 1.5 mM 2-DG in the larger volume (Table Ia). With 10,000 cells in either volume and estimating the dependence upon glucose utilization from the extent of inhibition of proliferation at 1.5 mM 2-DG , the relation between the percent-inhibition, volume and proliferative response to 1.5 mM 2-DG was similar, as was the response to 6 mM 2-DG and to MK 886 of 10^4 or $2x10^4$ cells in either volume (Table Ib).

Since clear-cut differences were not demonstrated with the path-length (depth) available in 96-well plates, to further examine the interplay between the oxygen diffusion path-length, 2-DG and relative hypoxia, we incubated 10^4 cells in scintillation counting vials, 5.5 cm in height with a cross-section area of 0.9 cm^2 under ambient conditions in increasing volumes of media. Increasing path-length from an average of 1.0 to 2.0 or 3.0 cm and inhibition of cellular replication by 2-DG were inversely correlated at 6.0 mM 2-DG (2 and 3 cm, reaching a limit) and 1.5 mM at a 3 cm depth (Table II). In effect, with increasing path-length, cells became more hypoxic and dependent upon glucose as 1.5 mM 2-DG was able increasingly to inhibit their proliferation and survival. Once HeLa cells were confluent, they did not overgrow the underlying monolayer.

To produce more definite degrees of hypoxia or virtual anoxia, we sealed lids of some plates with vaseline ("hypoxic" conditions) or cultured others under nitrogen in a sealed bell jar ("anoxic" conditions) to create oxygen-deficient environments (Table Ic,d; 10^4 cells/well). Viability as assessed from the O.D. 490 values under nitrogen or in plates with lids sealed with vaseline was not markedly altered from that seen when equal numbers of cells were incubated under aerobiosis (compared with Table Ia or Ib, 150 and 300 μl volumes). With "restricted" access to oxygen, inhibition due to 1.5 mM 2-DG increased under "hypoxia" (Table I b,d, 300 μl), indicating greater dependence of hypoxic cells on glucose and responsiveness to the inhibitor. Results comparing 150 and 300 μl aerobic and anoxic samples containing 10^4 cells initially (Figure 1b, c) did not provide any statistically significant differences. This possible anomaly was not studied further. Inhibition from 40 μM MK 886 was greatest under aerobiosis, less under hypoxia and least under anoxia, consistent with increasingly oxygen-deficient environments (Table Ic,d *versus* Ia).

Statistically significant differences ($p<0.05$) among these different categories are listed in Table III.

Interestingly, despite conditions intended to severely limit cellular replication and survival, in samples subjected both to inhibited glucose metabolism and reduced oxygen, cell viabililty was not seriously reduced. In these studies (Table Ib,c,d), the optical densities generated from the MTS assay correlated qualitatively with estimates of viable cells present, as assessed by microscopy. MK 886 strongly reduced proliferation and survival in cells under aerobiosis, consistent with its ability to inhibit mitochondrial function and induce programmed cell death in various cells (18, 19). Culture of cells under "hypoxia" indicated an increased dependence upon glucose for metabolic energy, as estimated by the greater inhibition by 1.5 mM 2-DG.

As we were not able to measure directly the concentrations of oxygen under these restrictive culture conditions, it was necessary to demonstrate that the procedures used to provide oxygen poor environments did achieve that result. Immunohistochemistry of Hif-1α provided supporting evidence for the effect of hypoxia or anoxia on cells incubated in multi-well slides sealed with vaseline or incubated under nitrogen (Figure 1). More extensive brownish-purple staining was present in nuclei of cells cultured under hypoxic (vaseline) and especially under "anoxic" (nitrogen) than under aerobiosis. When detached, the latter cells adopted a "fusiform" shape, others assuming a "sickle" cell configuration. Provided the concentration of oxygen is reduced, synthesis of Hif-1α protein should be increased. Hif-1α can be up-regulated in normal and cancer cells, and may be constitutively over-expressed (23). Under normoxic conditions, cells cultured for prolonged periods can express detectable Hif-1α, presumably as substrate and dissolved oxygen are depleted and ‘"toxic" products accumulate. No differences in staining between cells cultured aerobically in 96-well plates with 150 and 300 μl of medium per well were evident, nor was there much difference between cells incubated without primary antibody and cells cultured under aerobiosis (both not shown).

To further define these changes, Western blot analysis of Hif-1α protein concentrations was performed. Less immuno-reactive material estimated at about 120 kDa in extracts from "oxygenated" cells with progressively increased amounts in extracts from hypoxic and especially anoxic cells (Figure 2). In some experiments, aerobic culture for 3 days could increase the expression of Hif-1α, but to a lesser extent than cells cultured under anoxia or hypoxia.

Table I. *Percent inhibition of proliferation in 150 or 300 μl of medium by 6.0 or 1.5 mM 2-DG or by 40 μM MK-886 (MK) under aerobic, hypoxic or anoxic conditions. ("Emboldened No., p<0.05, see Table III).*

Ia. "Aerobic" conditions in N (10^4 in 150 μl) and 2N (in 300 μl) Cells / Well.

No.	O.D. control (490 nm)		Percent inhibition					
			150 μl			300 μl		
	150 μl	300 μl	6.0	1.5	MK	6.0	1.5	MK
1.	0.90	1.7	67	33	98	74	18	92
2.	0.80	1.5	74	35	95	65	15	60
3.	0.71	1.1	70	56	82	61	16	65
Avg.	0.80	1.43	70	**41**	91	67	**16**	**72**
Std. Dev.	0.09	0.30	3.5	12	8.5	6.6	1.5	17

Ib. "Aerobic" conditions with N (10^4) cells in 150 or 300 μl medium.

No.	O.D. control (490 nm)		Percent inhibition					
			150 μl			300 μl		
	150 μl	300 μl	6.0	1.5	MK	6.0	1.5	MK
1.	0.64	1.04	68	28	100	73	47	98
2.	0.79	1.10	**55**	23	85	53	30	89
3.	0.71	1.12	69	45	83	65	37	82
Average	0.71	1.08	64	32	89	63	38	89
Std. Dev.	0.08	0.04	7.8	11.5	9.3	10	8.5	8.0

Ic. Nitrogen ("anoxic") conditions.

No.	O.D. control (490 nm)		Percent inhibition					
			150 μl			300 μl		
	150 μl	300 μl	6.0	1.5	MK	6.0	1.5	MK
1.	0.75	1.10	52	33	44	46	34	30
2.	0.87	1.09	61	7	20	66	24	10
3.	0.95	1.16	67	27	20	62	10	27
4.	0.83	1.4	61	34	---	81	31	---
Avg.	0.85	1.18	60	23.5	**28**	64	29	**22**
Std. Dev.	0.08	0.14	6.2	13	14	14	3.5	11

Id. "Hypoxic" conditions.

No.	O.D. control (490 nm)		Percent inhibition					
			150 μl			300 μl		
	150 μl	300 μl	6.0	1.5	MK	6.0	1.5	MK
1	0.74	2.4^	63	30	77	72	56	17
2.	0.89	1.20	74	38	67	63	49	18
3.	0.64	1.25	68	45	54	79	50	39
4.	0.64	1.4	75	15	---	85	36	---
Average	0.75	1.34^ (2-4/2)	70	32	**66**	75	**48***	**22**
Std. Dev.	0.10	0.10	5.6	13	11	9.5	8	12

RPMI was diluted with PBS containing antibiotics, HEPES and fetal calf serum as described in the Materials and Methods section, resulting in a final glucose concentration of 3.3 mM. ^; 2+3+4/3=1.34.

Table II. *Effect of increasing depth of medium on the percent inhibition of proliferation by 2-deoxyglucose.*

	(% Change from control = 100%)					
	Depth of medium, cm:					
	0.5		1.0		2.0	
No.	(mM 2-DG)		(mM 2-DG)		(mM 2-DG)	
	6.0	1.5	6.0*	1.5	6.0*	1.5
1.	–5	+16	–24	+14	–40	+3
2.	–6	–8	–35	+10	–30	–22
3.	–16	+31	–33	+21	–27	–16
Average	**[-8.7 –/+6]**	**[+13 +/–4.9]**	[–31 –/+5.8]*	**[+15 +/–5.6]**	[–32 –/+6.8]*	**[–12 –/+0.7]***

Triplicate samples at each concentration were compared in each experiment and results from the 3 experiments averaged. Control = 100% and $*p \leq 0.05$ *versus* control.
As the depth of the medium increased, cells became more dependent on glucose, as reflected in the increased response to 1.5 mM 2-DG.

Table III. *Statistically significant differences ($p<0.05$) between samples incubated under different conditions, determined with Student's t-test.*

Aerobic/aerobic	1.5 mM 2-DG	150 / 300 µl (1a)
Aero/hypoxic	1.5 mM 2-DG	300 / 300 µl (1b/1d)
Hypoxic/hypo	1.5 mM 2-DG	150 / 300 µl (1d)
Aerobic versus	40 µM MK 886	300 / 300 µl (1a/b)
hypoxic or anoxic		150 / 150 µl (1c/1d)

Table IV. *HIF-1α transcription factor consensus sequence binding assay. Nuclear proteins from HeLa cells isolated using an Actif Motif nuclear protein kit were analyzed for their ability to bind to an Hif-1α consensus sequence as described in the Materials and Methods section. Optical densities at 450/650 nm were normalized to 100 µg protein.*

	OD 450/640 nm / 100 µg nuclear protein	
	No.1	No.2
Aerobic	0.006	0.28
Hypoxic	0.59	0.82
Anoxic	0.69	1.12

Finally, a transcription factor kit from Active Motif was used to demonstrate binding of Hif-1α protein extracted from nuclei of the 3 types of cells to its consensus sequence. Under the restrictive culture conditions, employing Pasteur plates completely filled with medium for the "hypoxic" samples, this early step of the activation of "upstream" Hif-1α signaling was observed, most prominently in nuclear extracts from cells incubated under nitrogen (Table IV).

These three estimations of Hif-1α formation served as surrogates for the direct measurement of oxygen concentration.

Discussion

Because of the imputed role of hypoxic cancer cells in therapeutic failure (7, 8, 24), we intended to compare the effect on HeLa cell replication and viability of reduced glucose consumption due to 2-DG under standard culture ("aerobic") conditions with different degrees of hypoxia or virtual anoxia, achieved by several means. We expected major reductions in cellular replication and extensive cell death with increasingly restrictive culture conditions. Initially, different volumes of medium in 96-well plates were used to examine the effect of increasing the oxygen diffusion path-length within the confines of the wells, without being able convincingly to do so. Unless the path length was considerably increased (Table II), the maximum depth of medium in wells of multi-well plates was insufficient and differences were complicated by considerations of the ratio between cell numbers and inhibitor. Despite supplementing this approach with more stringent conditions of hypoxia or virtual anoxia, to our surprise, significant replication and survival of these carcinoma cells continued during 48 to 72 h of culture.

In the initial experiments, with identical concentrations of reagents under aerobic conditions, 5% CO_2 and in most experiments, 10^4 cells in 150 µl or 10 to $20x10^3$ cells in 300 µl volumes, the extent of inhibition at 1.5 mM 2-DG in 300 µl was less than in 150 µl volumes. This difference disappeared when the same number of cells in both volumes or when 2N cells in 300 µl, containing 3.0 mM 2-DG were compared (latter not shown). This correlated with the ratio between the N and 2N cell-numbers and the fixed concentration of drug. In 96-well plates and different volumes of medium, the results depended upon cell numbers, concentrations of inhibitor, and their ratios, but the diffusion path-lengths available were not adequate to demonstrate the effect sought under the conditions employed unless the path-length was sufficiently increased (Table II).

In plates sealed with vaseline ("hypoxia") including a significant "dead" space above the wells or incubated under nitrogen ("anoxia"), employing ten thousand cells per well in both volumes, the percent inhibition at 1.5 mM 2-DG was not significantly different than that under normoxia. Differences related to volume and cell number (Table Ia, b) were reduced and MK 886 strongly suppressed proliferation in normoxia, less so with hypoxia and least under anoxia, consistent with an increasingly reduced availability of and dependence upon molecular oxygen. Increased reliance on

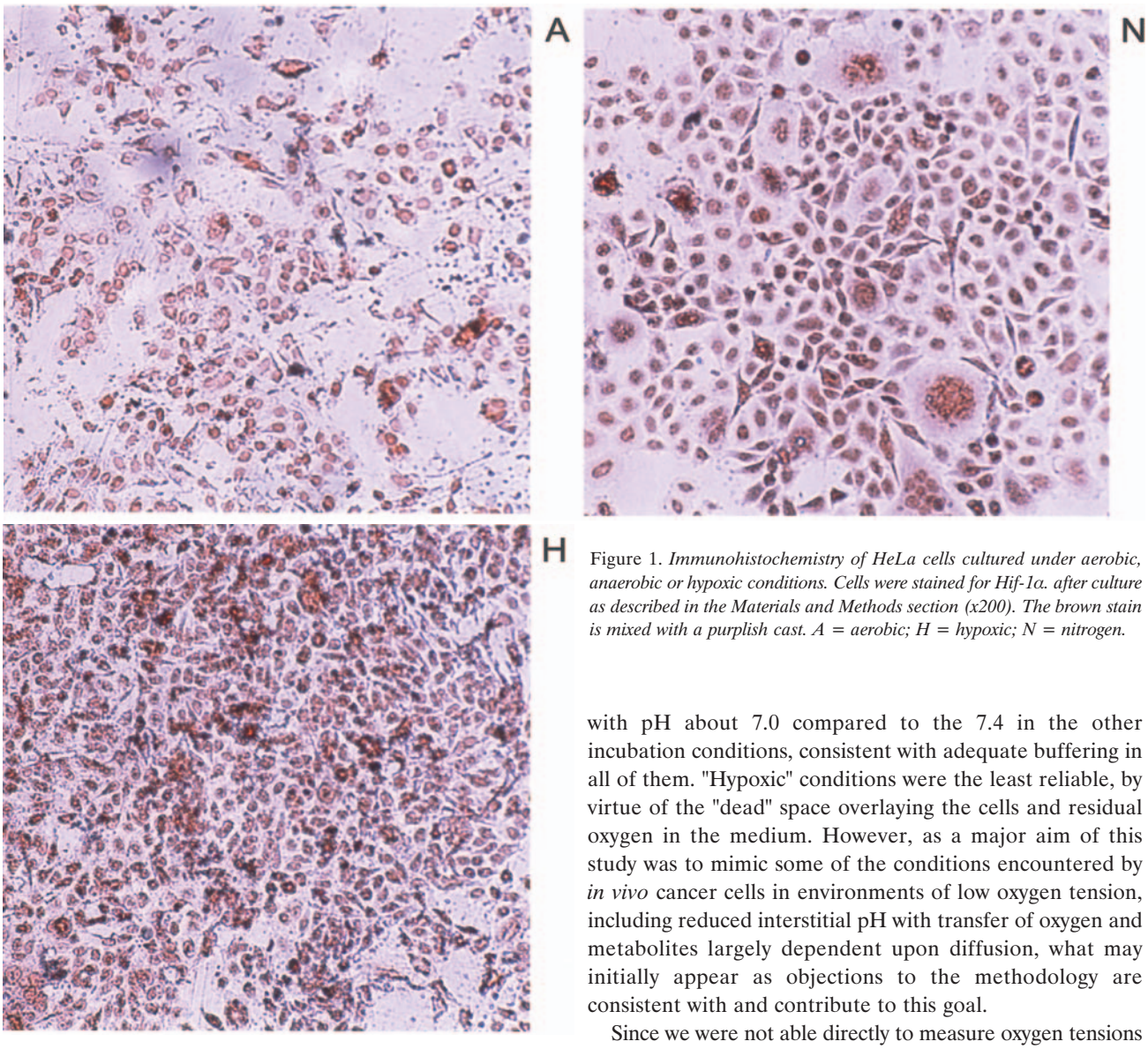

Figure 1. *Immunohistochemistry of HeLa cells cultured under aerobic, anaerobic or hypoxic conditions. Cells were stained for Hif-1α. after culture as described in the Materials and Methods section (x200). The brown stain is mixed with a purplish cast. A = aerobic; H = hypoxic; N = nitrogen.*

glucose was seen in 300 μl hypoxic samples; anoxic cells unexpectedly did not provide evidence for this increase, at least as estimated by the response to 1.5 mM 2-DG.

It is understood that the conditions employed cannot be entirely commensurate. Cells adhering to the bottom of the confining vessel under different overlying volumes of media and the extent of aerobiosis, hypoxia or anoxia differ in a number of aspects, not explicitly comparable. No attention was or easily could be paid to the redistribution or accumulation of CO_2 when incubations occurred under reduced oxygen tension. When assessed by phenol red in the medium or by pH paper, major differences were not found, although cells incubated under nitrogen produced a medium with pH about 7.0 compared to the 7.4 in the other incubation conditions, consistent with adequate buffering in all of them. "Hypoxic" conditions were the least reliable, by virtue of the "dead" space overlaying the cells and residual oxygen in the medium. However, as a major aim of this study was to mimic some of the conditions encountered by *in vivo* cancer cells in environments of low oxygen tension, including reduced interstitial pH with transfer of oxygen and metabolites largely dependent upon diffusion, what may initially appear as objections to the methodology are consistent with and contribute to this goal.

Since we were not able directly to measure oxygen tensions under these different conditions, comparison of the extent to which Hif-1α was expressed, whose concentration is known to increase under a sufficiently reduced oxygen-tension, was substituted. The Hif-1α transcription factor initiates cellular responses promoting cell survival and "malignant" biological behavior (24), including increased expression of anaerobic glycolysis, VEGF, Bil-2, glut-3, metalloproteinase inhibitor –13, plasminogen activator inhibitor, endothelin-2, GADD-45 and migration inhibitory factor (25), interacting with a variety of transcription factors (26).

The extent of survival and replication of cancer cells cultured in 96-well plates under unfavorable conditions was noteworthy. Despite efforts to reduce the availability of glucose and oxygen, especially stringent for cells inhibited by nitrogen and 2-DG, significant proliferation and survival occurred. *Ab initio*, and especially under hypoxia or anoxia, residual oxygen

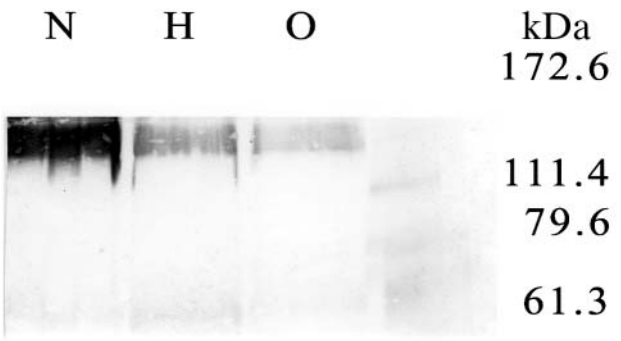

Figure 2. *Representative Western blot of proteins from "oxic", hypoxic or anoxic HeLa cells. Hela cells were cultured for 3 days under reduced oxygen, harvested manually, washed with PBS, suspended in Tris-buffer containing N-lauryl sarcosine and processed as described in the Materials and Methods section. O, oxic, H, hypoxic, N, anoxic.*

is present in the medium and in the "dead" space under the covers of plates sealed with vaseline. To reduce this dead-space, in some later experiments, Pasteur plates were filled with medium, increasing the amount of dissolved oxygen. Residual oxygen initially would support limited oxygen-dependent proliferation, consistent with the increasingly reduced inhibition from MK 886 under aerobic > hypoxic > anoxic conditions, paralleling the gradual reduction of the available dissolved oxygen. HeLa cells utilize glutamine as an alternate source of metabolic energy (17). Cells cultured with ethidium bromide to reduce the numbers of mitochondria and consequent oxidative phosphorylation survive when augmented with pyruvic acid, uridine and glucose (3, 27). Inability to identify all the sources of metabolic energy in MCF-7 cells (14) implies that additional sources and pathways of metabolic energy increase in response to metabolic stress (28). These could include lactate, fatty acids, amino acids, tri-acylglycerol, ketones, acetoacetate, *B*-hydroxybutyrate, and glycogen (14). A shift of energy sources to alternate metabolites and what would be most interesting, utilization of unidentified sources of metabolic energy entrained in oxygen-poor environments (14, 28) should also contribute to the survival of *in vivo* cancer cells in similarly restrictive environments.

It is noteworthy that MK 886, an agent known to be targeted to mitochondria, that inhibits ATP synthesis and increases the formation of reactive oxygen species (18-20), was, at the concentrations employed under conditions of aerobiosis, much more effective than 2-DG in reducing cell proliferation and survival, but this effectiveness declined with reduced access to oxygen The initial replication and significant survival of HeLa cells under restrictive conditions reflects this ability to adapt to an unfavorable energy environment. It also suggests that efforts to block mitochondrial function of hypoxic cells may not be very effective.

In summary, the intention of these experiments was to mimic *in vitro* an *in vivo* environment by inhibiting glucose metabolism in oxygen-poor cancer cells. The expectation was that their survival would be severely reduced and that the relative importance of glucose and oxidative metabolism could be estimated by the response to 2-DG and MK 886, respectively. Employing conditions that limit access to oxygen while measuring the response of cells to potential therapeutic agents may more faithfully predict their probable effect in patients.

There is evidence for the following considerations: (a) despite altering the aerobic / hypoxic / anoxic environments overlying attached HeLa cells while inhibiting proliferation with 2-DG, proliferation and survival were surprisingly resilient; (b) MK-886 strongly inhibited aerobic and to a lesser extent anaerobic (hypoxic) cell proliferation, while nitrogen-dependent anoxia was almost independent of this effect; (c) maintenance of cell proliferation and survival under adverse conditions must depend upon activation of facultative energy formation, in HeLa cells including the metabolism of glutamate and probably other substrates; (d) under these unfavorable conditions, the expected activation of a transcription factor, such as Hif-1α, is demonstrated, identifying the environment as deficient in oxygen; (e) to the extent that specific clones of hypoxic cancer cells up-regulate facultative, anaerobic, less directly molecular oxygen-dependent sources of metabolic energy, the effects of inhibiting glycolysis with 2-DG or related agents may be limited: however, recent studies of hexokinase associated with mitochondria and its release, use of bromopyruvate to inhibit the enzyme, and inhibitors of pyruvate dehydrogenase kinase and of ATP citrate lyase may provide additional routes to more effectively inhibit glucose utilization or induce programmed cell death in targets of interest (29); (f) multi-agent chemotherapy examined *in vitro*, especially if cellular energy formation is the major "target", should also include studies under hypoxic and virtually anoxic conditions.

Depending upon the agents selected and the metabolic capabilities of the targeted cells, limiting cell proliferation by reducing metabolic energy may have to be augmented by additional means, among others the suppression of Hif-1α and its hypoxia-induced pathways (8, 24, 26, 30). Finally, since both hypoxic cancer cells (7) and cancer "stem" cells (21) escape anticancer therapy, they may represent predominant novel treatment "targets" (31).

Acknowledgements

We thank the Seidel Family Trust and Dr. Jules Harris for their support. We thank Dr. A. Singh who kindly provided access to his photomicroscope and Dr. W. Alrefai for advice concerning Western blotting.

References

1 Holmuhamedov E, Lewis L, Bienengraeber M, Holmuhamedova M, Jahangir A and Terzic A: Suppression of human tumor cell proliferation through mitochondrial targeting. FASEB J *16*: 1010-1016, 2002.

2 Liu H, Hu YP, Savaraj N, Priebe W and Lampidis TJ: Hypersentization of tumor cells to glycolytic inhibitors. Biochemistry *40*: 5542-5547, 2001.

3 Desjardins P, Frost E and Morais R: Ethidium bromide-induced loss of mitochondrial DNA from primary chicken embryo fibroblasts. Mol Cell Biol *5*: 1163-1169, 1985.

4 Nirenberg MW and Hogg JF: Inhibition of anaerobic glycolysis in Ehrlich ascites tumor cells by 2-deoxy-D-glucose. Cancer Res *18*: 518-521, 1958.

5 Maschek G, Savaraj N, Priebe W *et al*: 2-Deoxy-D-glucose increases the efficacy of adriamycin and paclitaxal in human osteosarcoma and non-small cell lung cancers *in vivo*. Cancer Res *64*: 31-34, 2004.

6 Gatenby RA and Gillies RJ: Why do cancers have high aerobic glycolysis? Nat Rev Cancer *4*: 891-899, 2004.

7 Graeber TG, Osmanian C, Jacks T *et al*: Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. Nature *379*: 88-91, 1996.

8 Brown JM: Hypoxic cells. A target for selective cancer therapy. Cancer Res *59*: 5863-5870, 1999.

9 Lu H, Forbes RA and Verma A: Hypoxia-induced factor 1 activation by aerobic glycolysis implicates the Warburg effect in carcinogenesis. J Biol Chem *277*: 23111-23115, 2002.

10 Seagroves TN, Ryan HE, Lu H *et al*: Transcription factor HIF-1 is a necessary mediator of the Pasteur effect in mammalian cells. Mol Cell Biol *21*: 3436-3444, 2001.

11 Matoba S, Kang J-G, Patino WD *et al*: p53 regulates mitochondrial respiration. Science *312*: 1650-1653, 2006.

12 Robin ED, Murphy BJ and Theodore J: Coordinate regulation of glycolysis by hypoxia in mammalian cells. J Cell Physiol *118*: 287-290, 1984.

13 Elstrom RL, Bauer DE, Buzzai M *et al*: AKT stimulated aerobc glycolysis in cancer cells. Cancer Res *64*: 3892-3899, 2004.

14 Guppy M, Leedman P, Zu X and Russell V: Contribution by different fuels and metabolic pathways to the total ATP turnover of proliferating MCF-7 breast cancer cells. Biochem J *364*: 309-315, 2002.

15 Sutherland RM: Cell-environment interaction in tumor microregions: The multi-spheroid model. Science *240*: 177-184, 1986.

16 Smith RAJ, Porteous CM, Coulter CV and Murphy MP: Selective targeting of an antioxidant to mitochondria. Eur J Biochem *263*: 709-716, 1999.

17 Reitzer LJ, Wice BM and Kennell D: Evidence that glutamine, not sugar, is the major energy source for cultured HeLa cells. J Biol Chem *254*: 2669-2676, 1979.

18 Anderson KM, Seed T, Jajeh A *et al*: An *in vivo* inhibitor of 5-lipoxygenase, MK 886 at micromolar concentration induces apoptosis in U937 and CML cells. Anticancer Res *16*: 2589-2600, 1996.

19 Anderson KM, Alrefai WA, Dudeja PK *et al*: Increased cytosol Ca^{2+} and type 1 programmed cell death in Bcl-2-positive U937 but not in Bcl-2-negative PC-3 and Panc-1 cells induced by the 5-lipoxygenase inhibitor MK 886. Prost Leuko Ess F A *66*: 443-452, 2002.

20 Gugliucci A, Ranzato L, Scorrano L *et al*: Mitochondria are direct targets of the lipoxygenase inhibitor MK 886. A strategy for cell killing by combined treatment with MK 886 and cyclooxygenase inhibitors. J Biol Chem *227*: 31789-31795, 2002.

21 Jones RJ, Matsui WH and Smith BD: Cancer stem cells: Are we missing the target? J Nat Cancer Inst *96*: 583-585, 2004.

22 Wu W, Welsh MJ, Kaufman PB and Zhang HH: Analysis of gene expression at the protein level. Chapt. 7. *In*: Methods in Gene Technology, CRC Press, pp. 241-258, 1997.

23 Zhong H, De Marzo AM, Laughner E *et al*: Overexpression of hypoxia-inducible factor 1a in common human cancers and their metastases. Cancer Res *59*: 5830-5835, 1999.

24 Semenza GL: HIF-1 and tumor progression: pathophysiology and therapeutics. Trends Mol Med 8 (4 suppl): S62-67, 2002.

25 Koong AC, Denko NC, Hudson KM *et al*: Candidate genes for the hypoxic tumor phenotype. Cancer Res *60*: 883-887, 2000.

26 Huang LE and Bunn HF: Hypoxia-inducible factor and its biomedical relevance. J Biol Chem *278*: 19575-19578, 2003.

27 King MP and Attardi G: Human cells lacking mDNA: Repopulation with exogenous mitochondria by complementation. Science *246*: 500-503,1989.

28 Gabaldon T and Huynen MA: Reconstruction of the proto-mitochondrial metabolism. Science *301*: 609, 2003.

29 Garber Ken: News. Energy deregulation: Licensing tumors to grow. Science *312*: 1158-1159, 2006.

30 Chau NM, Rogers P, Aherne W *et al*: Identification of novel small molecule inhibitors of hypoxia-inducible factor-1 that differentially block hypoxia-inducible factor-1 activity and hypoxia-inducible factor 1α induction in response to hypoxic stress and growth factors. Cancer Res *64*: 4918-4928, 2005.

31 Anderson KM, Tsui P, Guinan P and Rubenstein M: To what extent are the "sets" of biologic properties expressed by hypoxic cancer cells and by cancer "stem" cells equivalent, intersecting, disjoint or complementary? Some implications for the design of cancer therapies. Med Hypo, in press, 2006.

Received August 18, 2006
Accepted September 5, 2006