In Vitro Effects of Dichloroacetate and CO_2 on Hypoxic HeLa Cells

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Abstract. *HeLa and PANC-1 cells were exposed to conflicting signals promoting anaerobic or aerobic energy-generating processes and their viability, cell numbers and the ability of HeLa cells to form colonies were assessed. Under conventional aerobic cell culture with 5% CO_2, dichloroacetate (DCA), an inhibitor of the enzyme pyruvate dehydrogense kinase with subsequent stimulation of pyruvate dehydrogenase that redirects energy metabolism toward the Kreb cycle, reduced HeLa and PANC-1 cellular proliferation and viability. With nitrogen-induced hypoxia, the number of control cells and cells cultured with 12.5 mM DCA paradoxically was greater than that of normoxic controls under similar conditions. A higher medium pH of cells cultured under nitrogen contributed to these differences. In 96-well experiments, 95% nitrogen with 5% CO_2 reduced the numbers of hypoxic cells and medium pH toward that of the aerobic controls, with retention of the DCA-induced hypoxic compared to normoxic cell numbers. The media of these cells cultured with DCA still exhibited an increased pH. Increased hypoxia-inducible factor 1, alpha subunit (HIF1A) mRNA expression in hypoxic HeLa cells and their greater reliance on D-glucose for metabolic energy confirmed the reliability of the incubation conditions. Compared with normoxic cells, hypoxic cells initially increased their synthesis of ATP, but once proliferation ceased, this no longer closely correlated with cell numbers. Type 1 apoptosis, which was somewhat greater in hypoxic than normoxic cells, contributed to hypoxia and DCA-induced cell death. Colony counts of hypoxic, DCA-inhibited cells subsequently switched to normoxia exceeded those of similarly treated normoxic DCA cells. Despite inhibition in certain hypoxic environments of pyruvate dehydrogenase kinase by DCA and its contribution to increased cellular apoptosis and necrosis, hypoxic cells generally outnumbered normoxic control cells, as did hypoxic DCA-treated cells compared with comparable DCA-treated normoxic cells. Since in vivo hypoxic cells are considered a major factor contributing to therapeutic failure, and as DCA redirects energy metabolism toward the more energy efficient Kreb citric acid cycle, associated with increased medium (and inferred cellular) pH, similar circumstances in vivo could promote proliferation and survival of hypoxic cell clones with the potential for developing unwanted properties.*

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Dichloroacetate (DCA) reduces phosphorylation of pyruvate dehydrogenase (PDH) by pyruvate dehydrogenase kinase (PDK), increasing the activity of PDH, with greater formation of acetyl-CoA and redirecting energy metabolism away from glycolysis and toward Kreb cycle aerobic mitochondrial oxidative phosphorylation (1). DCA has a therapeutic role in metabolic acidosis (2) and in a number of inherited mitochondrial-dependent disorders (3). PDK occupies a central role in down-regulating mitochondrial oxygen consumption (4, 5).

Recently, DCA was reported to inhibit proliferation and induce apoptosis of human cancer cells exhibiting specific properties, both *in vitro* and *in vivo* (6). Unlike non-malignant cells, proliferation of cancer cells including A572 lung and MCF-7 breast lines, expressing high mitochondrial membrane potentials and reduced K^+ channel (Kv1.5) activity, was inhibited by DCA. As a result of reduced mitochondrial PDK activity, metabolism from oxygen-deficient, less aerobic glycolysis with increased formation of lactic acid was redirected toward metabolism *via* mitochondrial oxidation. This was accompanied by reduced mitochondrial membrane potential, increased formation of H_2O_2 and activation of Kv1.5 potassium channels by a nuclear factor of activated T-cells (NFAT)-dependent mechanism. In both *in vitro* and *in vivo* studies of A549 non-small cell lung cancer cells transplanted in nude mice, DCA reduced proliferation and accompanied by the aforementioned changes in mitochondrial function, increased apoptosis and limited tumor growth. Studies with siRNA directed against PDK2 produced comparable effects.

Some of these features suggest an interesting parallel with an earlier study of transformed and non-transformed mouse embryo fibroblasts subjected to hypoxia (7). Following hypoxia-induced G_0/G_1-arrest, malignantly transformed fibroblasts entered apoptosis but non-transformed controls did not. Apoptosis required an acidic environment and when uncoupled from this, the viability and clonogenicity of hypoxic cells increased. Hypoxia up-regulated P53 activity that was associated with acidosis but the reverse occurred in hypoxia without acidosis. The conclusion was that this latter response to hypoxia could lead to drug resistance and reduced cell destruction by apoptosis. Evidence was also cited that in addition to regions that were more acidic, hypoxic regions in solid tumors could contain more alkaline regions (8). Taken altogether, hypoxic regions associated or not with putative alkaline regions of a solid tumor contain cells more likely to resist therapy and evolve variant forms (9, 10).

Based on this information, we wondered what the initial interplay would be between comparatively anaerobic, cytosol-dependent glycolysis induced by hypoxia and the enhancement of a countervailing DCA-induced aerobic, mitochondria-dependent energy metabolism. The latter state should tend to inhibit the former, but with what outcome on hypoxic cancer cell survival, proliferation and clonogenicity was arguable. In addition, we wanted to confirm that the MTS assay applied to hypoxic cells reflects cell viability/number rather than an adventitious change in the regulation of dehydrogenase enzymes, among which some were likely responsible for reducing the MTS substrate.

Materials and Methods

Cell culture. HeLa cervical or PANC-1 pancreatic cancer cells obtained from the American Type Cell Collection , Betheda, MD, USA were separately cultured for 48 or 72 h in 96-well microtiter plates at 37˚C with 5% CO_2 ("normoxic"conditions). Hypoxic conditions were defined as the atmosphere present when 96-well microtiter plates or flasks were incubated in a sealed bell jar humidified with 10-15 ml water and containing a predominant atmosphere of nitrogen in early studies or subsequently with 95% nitrogen and 5% CO_2, the latter with an imputed O_2 concentration of about 10 ppm (7). The culture medium, totaling some 12 ml in 40 wells containing 0.3 ml medium per well can be estimated to contain about 0.2 mM oxygen (11) and about 1.28 mM CO_2 at 37˚C, the latter however in plasma (12). The mole fractions of oxygen and CO_2 in water at 25˚C are approximately 2.3×10^{-5} and 6.15×10^{-4} (13). Our intention was to exchange the atmosphere within the jar without significantly depleting oxygen or carbon dioxide dissolved in the RPMI-1640 medium. RPMI-1640 medium contained GlutaMax, 10% fetal bovine serum (FBS), 50 U/ml penicillin G, 0.05 mg/ml streptomycin, 25 mM HEPES, 23 mM $NaHCO_3$ and 200 mM D-glucose, the latter two included in the original RPMI. In most 96-well experiments, RPMI without phenol red was used, in order to reduce the background. Cells were usually sub-cultured one or two days before use, detached with 0.25% trypsin-0.03% EDTA and their initial number and viability determined using a hemocytometer by exclusion of trypan blue. Each well contained 5,000 viable cells in a 300 μM volume. After culture, cell proliferation and viability were measured with an MTS-related assay at 490 nm, according to instructions provided with a commercial kit (Promega, Madison, WI, USA), and cell densities compared by direct measurement of control cells and those cultured with 12.5 mM DCA as described in Table IV. 2-Doxy-D-glucose (2-DG) was prepared in complete RPMI at a final concentration of 25 mM. DCA (GMW 127, density 1.567 g/ml, 99% stock), freely soluble in complete medium, was first neutralized to pH 7.4 before addition of FBS; considerable NaOH was required to neutralize this strong acid.

After incubation, in order to avoid the rapid rise in pH before its accurate measurement, the pH of normoxic and hypoxic cell media were immediately compared visually, when phenol red was present and with pH paper.

RT-PCR of hypoxia inducible factor 1, alpha subunit (HIF1A). A Superscript III one-step RT-PCR system (Invitrogen, Carlsbad, CA, USA) was used to compare *HIF1A* mRNA expression in normoxic and hypoxic cells. An *HIF1A* primer set was obtained from Real Time Primers, Elkins Park, PA, USA. The *HIF1A* primer contained a 164 bp fragment with a suggested temperature cycle of 95˚-58˚C degrees for 50 cycles. The forward primer was: 5'-GCT GAT TTG TGA ACC CAT TC-3' and the reverse primer was 5'AAA TTG AGC GGG CTA AAA GT-3'. The 200 base pair DNA standard was from Invitrogen.

Determination of ATP. ATP formation, as a surrogate assay of cell proliferation, was measured with a Promega Luminescent Cell Viability ATP kit, employing luciferase according to the accompanying instructions. The assay should serve as a second estimate of viable cell number independent of possible changes in dehydrogenase activity.

Contribution to cell death from apoptosis identified by flow cytometry. Following incubation with or without DCA for 24 or for 96 h, cells were examined with an Immunochemistry Technologies (Bloomington, MN, USA) Flow Cytometric Carboxyfluorescein (Fluorescein Inhibitor of Caspases, FLICA) Apoptosis Detection Kit according to the accompanying instructions. FAM-VAD-FMK is a carboxyfluorescein (FAM) derivative of valyalanylaspartic acid (VAD) and fluoromethyl ketone (FMK), a polycaspase inhibitor. Measurements were performed in the Flow Cytometry Laboratory at the University of Illinois Medical Center in Chicago, IL, USA.

Clonogenicity assays. After incubation of normoxic and hypoxic cells with or without 12.5 mM DCA, 500 or 1,000 viable cells from DCA-treated samples and normoxic controls were cultured in 25 ml flasks for up to 15 days. Once colonies of 25 or more cells appeared, the medium was removed, then the cells stained with 0.1% crystal violet in 90% ethanol and counted.

Chemicals and FBS were obtained from Sigma Biochemicals, St. Louis, MO, U.S.A.

Results

Initial effect of DCA on viability and proliferation of cells under normoxic or hypoxic conditions, as modulated by CO_2 and pH. We first compared cells under conventional normoxic conditions of ambient atmosphere with 5% CO_2 with cells cultured under nitrogen for 72 hours. Despite the

presence of 25 mM HEPES, 25 mM $NaHCO_3$ and 10% FBS, the pH of the medium from the hypoxic cultures rose to about 7.8, compared to the 7.3 of normoxic cells (Table IA). The increase in pH of hypoxic cell media was associated with greater cell proliferation of the hypoxic cells, especially at 12.5 mM DCA compared with normoxic DCA cells. Normoxic cells around pH 7.4 or less, with or without DCA were inhibited to a greater extent. Cells were next incubated in an ambient atmosphere (20% oxygen and CO_2 of 0.03%) and compared with cells under nitrogen alone (Table IB). Both media became alkaline, with more cells present in the ambient control than the hypoxic sample. The response to DCA in both was comparable. To avoid the influence of artifactual alkaline pH on cell proliferation and identify the respective contribution of hypoxia or DCA to proliferation, normoxic cells under 5% CO_2 were compared with cells under N_2/5% CO_2 (Table IC). The pH of DCA-containing samples was still more alkaline, with pHs of about 7.5, compared to control pHs of around 7 to 7.3. Control cell numbers were similar under both conditions but hypoxic cells cultured with 12.5 mM DCA were present in greater numbers relative to their normoxic controls.

To further confirm the effect of 5% *versus* 0.03% CO_2 on proliferation, cells were cultured under normoxic (5% CO_2), ambient (0.03% CO_2) and hypoxic (with estimated O_2 and CO_2 concentrations) conditions for 48 hours and cell numbers measured with the MTS assay (Table ID). As seen previously, the number of ambient and hypoxic cells exceeded normoxic cell numbers.

The MTS assay has been used to study the viability and proliferation of hypoxic cells (14, 15). Compared with normoxia, hypoxia might induce an artifact by altering the activity of oxidation/reduction enzymes such as NADPH quinone acceptor oxidoreductase (EC1.6.99.2, also known as menadione reductase, DT diaphorase) and cytochrome P450, on which the MTS assay is based (16).

To consider this question further, in several experiments we compared the number of viable normoxic or hypoxic cells after culture with 12.5 mM DCA in 96-well and other culture vessels, keeping in mind that the ratio of drug to cells sharply declines with different cell inputs (Table II). These results mirror those from the MTS assay from 96-well plate studies, with generally greater numbers of viable cells recovered from hypoxic cell cultures under these restrictive conditions.

In view of the more limited response of PANC-1 cells and their tendency to clump, HeLa cells were used in most of the subsequent experiments.

Concentration of ATP under hypoxic culture conditions. To provide an additional measure of cell proliferation, an assay which depends upon firefly luciferase to measure relative concentrations of ATP was employed. The result of such an

Table I. *Optical densities (490 nm) of cells under different conditions of oxygenation and DCA as a measure of cell number. One of 3 representative experiments. N=5 for each data point, ± is the standard deviation.*

A. Normoxia (ambient O_2 + 5%CO_2) versus Nitrogen.

	HeLa		PANC-1	
	Normoxia	Nitrogen	Normoxia	Nitrogen
Control	1.5±80.10	2.1±0.09	0.45±0.02	0.74±0.10
DCA mM				
1.6	1.6±0.07	2.34±0.14	0.44±0.03	0.62±0.04
3.18	2.0±0.26	2.44±0.07	0.51±0.06	0.73±0.11
6.25	2.0±0.27	2.34±0.20	0.44±0.05	0.70±0.11
12.5	0.24±0.01	0.99±0.11	0.24±0.04	0.48±0.04
25	0.19±0.09	0.28±0.01	0.20±0.02	0.29±0.02

B. Ambient (20% O_2, 0.03% CO_2) *versus* nitrogen (trace O_2 and CO_2) (final pH of both=7.8). One of 3 experiments with N=5, ± is the standard deviation.

	HeLa		PANC-1	
	Ambient	Nitrogen	Ambient	Nitrogen
Control	1.11±0.08	0.56±0.03	0.80±0.03	0.44±0.02
DCA mM				
1.6	1.03±0.13	0.58±0.04	0.83±0.06	0.43±0.02
3.18	0.92±0.20	0.51±0.05	0.67±0.03	0.46±0.04
6.25	0.82±0.09	0.60±0.04	0.70±0.01	0.46±0.09
12.5	0.43±0.05	0.45±0.05	0.45±0.04	0.45±0.05
25	0.20±0.01	0.19±0.01	0.28±0.05	0.18±0.02

C. Normoxia (ambient O_2 + 5% CO_2) *versus* 95% N_2-5% CO_2.

	HeLa		PANC-1	
	Normoxia	N_2-CO_2	Normoxia	N_2-CO_2
Control	0.79±0.04	0.79±0.04	0.64±0.06	0.63±0.04
DCA mM				
1.6	0.57±0.08	0.90+/-0.05	0.40±0.06	0.66±0.07
3.18	0.43±0.05	0.74±0.05	0.45±0.09	0.77±0.12
6.25	0.39±0.05	0.82±0.05.	0.47±0.09	0.64±0.07
12.5	0.39±0.06	0.71±0.08	0.19±0.02	0.41±0.05
25	0.30±0.03	0.56±0.04	0.26±0.06	0.26±0.05

D. Normoxic, hypoxic and ambient (20% O_2, 0.03% CO_2) cells cultured for 48 h. The pH of ambient and of nitrogen-cultured HeLa cell media was 7.5 compared to 7.2 for the normoxic cell media. Optical densities were measured at 490 nm, as above with N=3.

Normoxic	0.39±0.09
Ambient	0.70±0.08
Hypoxic	0.57±0.08

Table II. *Effect of culture vessels on viability of HeLa cells cultured with 12.5 mM DCA under normoxia or hypoxia. Cells were cultured in different vessel types under the indicated conditions, supernatants aspirated, cells washed with normal saline, trypsin-EDTA added and cells detached. Where appropriate, cells were combined from similar 96-well samples, centrifuged, taken up in small volumes of medium, trypan blue added and cell counts performed. Fewer than 1% of cells were trypan blue positive.*

	Cells/ml		
	Normoxic	Hypoxic	Ratio (H/N)
96-Well plates			
1	48,800	95,400	1.9
2	28,800	42,200	1.5
25 cm^2 Flasks			
3	310,000	520,000	1.6
4	230,000	480,000	2.1
6-Well plates			
5	141,000	180,000	1.3

Table III. *ATP synthesis under normoxic and hypoxic conditions. Twenty thousand cells per well were incubated under normoxia or hypoxia, with or without 12.5 mM DCA for 24h (1) 48 h (2) or 96 h (3) before ATP was measured in a Berthold luminometer, using a Promega luciferase assay according to the instructions provided. Measurements are in relative light units (RLU).*

	Normoxia		Hypoxia	
	RLU	O.D. 490 nm	RLU	O.D. 490 nm
1	39,862±4684	1.26	58,903±7375	1.46
+DCA	27,954±130	0.92	52,335±791	1.12
2	195,040±17,909	0.93	210,342±4,796	1.14
+DCA	146,530±4698	0.79	169,612±3476	0.84
3	135,201±8753.	2.35	169,341±1545	1.06
+DCA	223,603±4294	1.24	297,374±17,112	1.40

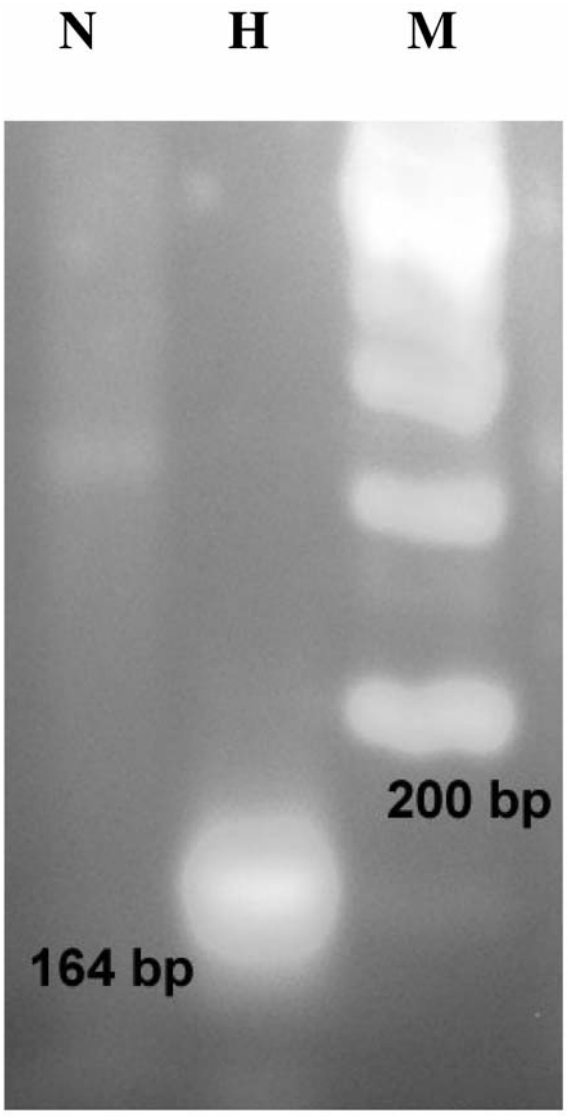

Figure 1. *RT-PCR of HIF1A in hypoxic and normoxic cells. N, Normoxic; H, hyopoxic; M, DNA standards.*

assay should be independent of any effect of hypoxia on oxidation-reduction enzymes contributing to the reduction of MTS. Based on the relative light unit (RLU) values, the number of hypoxic control cells exceeded that of the normoxic cells, as did the numbers of DCA-treated hypoxic cells compared with DCA-treated normoxic cells (Table III). The results with control normoxic and hypoxic cells correlate with those of the MTS assay, provided measurements are taken when cells are in cycle. Subsequent metabolism of ATP by cells no longer proliferating could obscure the initial results (Table III). Under these conditions, the ATP concentrations reflected in the RLU values of inhibited cells need not correlate with their estimated density as determined by the MTS assay.

Expression of HIF1A mRNA in normoxic and hypoxic cells. To confirm that incubations in a sealed bell jar including a predominant atmosphere of 95% N_2 and 5% CO_2 provided sufficiently hypoxic conditions, expression of *HIF1A* mRNA was determined under normoxic and hypoxic conditions (Figure 1). Comparable inputs of RNA from normoxic and hypoxic cells yielded a strong signal with RNA from the hypoxic sample at about 164 bp, but no signal was apparent with normoxic RNA, at least not in the preparation used. The appearance of *HIF1A* mRNA is consistent with the increased presence in Western blots of HIF1A protein from hypoxic cells (17). In those experiments, a lesser amount of the protein was present in normoxic cells.

Table IV. *Combined effect of DCA and 2-deoxy-D-glocose and hypoxia on cell proliferation. Normoxic cells compared with cells in N_2.*

	Normoxic	Hypoxic
Control	0.63±0.02	1.44±0.04
Control + 2DG	0.34±0.04	0.43±0.04
% Inhibition	–47	–70
6.25 mM DCA	0.71±0.07	0.78±0.12
6.25 mM +2DG	0.39±0.03	0.32±0.02
% Inhibition	–45	–59
12.5 mM DCA	0.31±0.02	0.80±0.03
12.5 mM+2DG	0.24±0.03	0.28±0.01
% Inhibition	–23	–65
25 mM DCA	0.26±0.02	0.29±0.03
25 mM +2DG	0.23±0.02	0.28±0.02
% Inhibition	–12	–4

The effect of 2-deoxy-D-glucose on cell proliferation under normoxia or hypoxia. and the response to DCA. To confirm an increased contribution of D-glucose to anaerobic glycolysis under hypoxia compared with aerobic glycolysis in normoxia, we measured the effect of 2-DG on the proliferation of cells cultured with DCA in N_2/CO_2 (Table IV). Hypoxic control cells cultured with 25 mM 2-DG exhibited a greater reduction in O.D 490 nm, compared with normoxic cells, as did cells with 12.5 mM or less DCA, consistent with increased dependence of hypoxic cells on glucose.

The nature of cell death associated with DCA under these conditions. With the conditions employed in these experiments, cell death represents one feature of the response to DCA (6). We cultured 2.5×10^6 cells in 75 cm^2 flasks under normoxia or hypoxia, including samples with 12.5 mM DCA for 24 and 96 h. Detection of type 1 apoptosis demonstrated apoptotic cell death in both hypoxic and normoxic, DCA-inhibited samples (Figure 2, Table V). Culture with DCA was associated with greater numbers of necrotic cells. Whether necrotic cells were generated initially by apoptosis and also from other modes of cell death, including autophagy, necrosis (as a discrete process), senescence and mitotic catastrophe was not examined further (18).

Clonogenicity of cells switched from hypoxia to normoxia. The clonogenicity of 500 or 1,000 viable cells from normoxic cells±DCA or from hypoxic±DCA populations, first cultured in 95% N_2–5% CO_2 and subsequently transferred to normoxic conditions for up to 18 days was compared (Table VI, Figure 3). In the majority (3/4) of experiments, the number of normoxic cell colonies exceeded that of the formerly hypoxic colonies, while formerly hypoxic DCA-containing colonies were somewhat more numerous than normoxic formerly DCA-containing colonies.

Table V. *Identification of apoptosis as a contributor to HeLa cell death. Approximately 2.5×10^6 HeLa cells were incubated with or without 12.5 mM DCA for either 24 or 96 h, then labeled with FAM- VAD-FMK and propidium iodide, as described in the Materials and Methods, and flow studies performed at the Flow Cytometry Laboratory of the University of Illinois in Chicago.*

	Proportion of cells (%)			
	Normoxic	Normoxic +DCA	Hypoxic	Hypoxic +DCA
24-h				
Early apoptosis	1.5	1.5	1.6	1.1
Late apoptosis	1.7	1.0	2.6	1.2
Viable cells	94.0	93.0	88.0	90.0
Necrotic cells	3.8	4.5	7.6	7.8
96-h				
Early apoptosis	1.3	2.5	2.4	2.5
Late apoptosis	2.1	8.8	2.9	7.1
Viable cells	64.0	50.0	70.0	54.0
Necrotic cells	33.0	38.0	26.0	36.0

Table VI. *Colony count of cells under normoxic and hypoxic conditions, incubated or not with DCA and subsequently cultured under normoxic conditions. Cells were incubated as described previously, media removed, cells detached and 500 (#3) or 1,000 cells per flask cultured in RPMI under standard culture conditions for up to 18 days or until significant numbers of colonies formed.*

	Number of colonies			
	#1	#2	#3	#4
Normoxic	310	108	5	91
Normoxic + 12.5 mM DCA	20	51	7	39
Hypoxic	22	78	2	93
Hypoxic + 12.5 mM DCA	77	63	18	54

The result with normoxic cells could be an anomaly. Hypoxic murine tumor cells over-replicate DNA under hypoxic conditions (19) while prototrophic yeast cells replicate DNA during a reductive, glycolytic phase of metabolic activity (20).

Discussion

The original intention was to compare the response of normoxic and hypoxic cells exposed to DCA, an agent known to redirect cellular metabolism toward mitochondria-dependent aerobic glycolysis. Cells were briefly exposed initially to N_2 but subsequently to N_2 –5% CO_2 sufficient to exchange the atmosphere in the bell jar but considered insufficient to replace dissolved oxygen in the medium and the

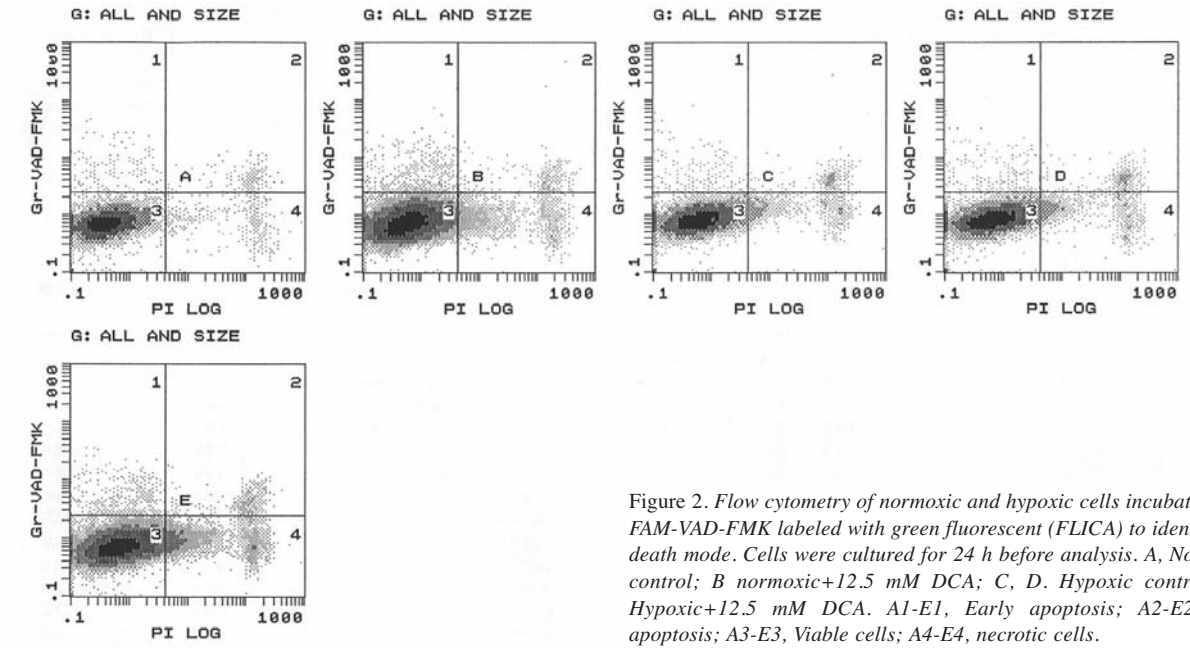

Figure 2. *Flow cytometry of normoxic and hypoxic cells incubated with FAM-VAD-FMK labeled with green fluorescent (FLICA) to identify cell death mode. Cells were cultured for 24 h before analysis. A, Normoxic control; B normoxic+12.5 mM DCA; C, D. Hypoxic controls; E, Hypoxic+12.5 mM DCA. A1-E1, Early apoptosis; A2-E2, Late apoptosis; A3-E3, Viable cells; A4-E4, necrotic cells.*

10 to 15 ml water included in the bell jar to maintain humidity. Any oxygen-dependent aerobic metabolism utilized this dissolved oxygen and the limited residual oxygen present in the N_2/CO_2 mixture. Estimates of approximate concentrations of residual O_2 and CO_2 in the bell jar, media and added water for maintenance of humidity were mentioned previously.

Several criticisms are apparent. The inability to measure concentrations of residual oxygen and imprecision in the measurement of pH due to its rapid increase in the ambient atmosphere contributed to some variation in the results. The extent to which incubation conditions affect HeLa cell voltage-dependent Kv1.5 (or other) potassium channel activity was not established. In fact, the requirement for higher concentrations of DCA to inhibit proliferation might suggest involvement of mechanisms unrelated to or in addition to an altered potassium channel activity and its regulation. In some experiments, DCA at low concentrations increased proliferation of control normoxic cells by 10% to 20%, a result not studied further. The extent, if any, to which the NaOH required to neutralize the stock 100 mM DCA might contribute to DCA-associated alkalinity is not established. The role of P53 which also affects the balance between the glycolytic and respiratory pathways (21, 22) and is mutated in many types of cancer was not examined

Finally, results based on experiments employing non-physiological pCO_2 and unknown pO_2 concentrations raise some questions as to their extrapolation *in vivo*. Nevertheless, with the conditions employed, proliferation of hypoxic cells exceeded that of cells cultured under the traditional normoxic culture conditions of ambient atmosphere supplemented with 5% CO_2.

Numerous examples of altered cell growth and proliferation with both non-malignantly transformed cells and cancer cells cultured with DCA and of hypoxic cells have been noted (23-35). Under hypoxic conditions, DCA may provide an apparent limited proliferative stimulus, presumably from augmented mitochondrial oxidative metabolism of glucose and other substrates capable of conversion to acetyl-CoA. Reduced apoptosis, potentially contributed to by an up-regulation of BCL-2 (36, 37) was not excluded as a contributing feature.

When the confounding effect of pH was largely reduced with the use of the N_2/CO_2 mixture, limited exposure of hypoxic cells to 12.5 mM DCA resulted in greater survival, compared with DCA-exposed normoxic cells, judged with an MTS assay. This could occur in experiments in which control normoxic and hypoxic MTS results were comparable (*e.g.* Table IC, Table III, #1, 2). With prolonged culture, proliferation of normoxic cells measured with the MTS assay can exceed that of hypoxic cells (Table IV, #3, 96 h). Results can depend upon the geometry of the vessels employed and kinetics of proliferation, since once confluence is achieved, HeLa cells do not accumulate further under either condition. Interestingly and possibly crucially, 0.5 mM DCA increased the pH of A549 cells cultured under conventional conditions, accompanied by reduced lactic acid as aerobic metabolism increased (6). Characteristically, media from hypoxic, DCA-containing cultures were more alkaline than their respective controls.

As presented in Table IB, the pH of media of cells cultured in an ambient atmosphere (20% O_2, 0.03% CO_2) and cells cultured under N_2 both increased to 7.8, with greater proliferation of the cells under ambient compared with

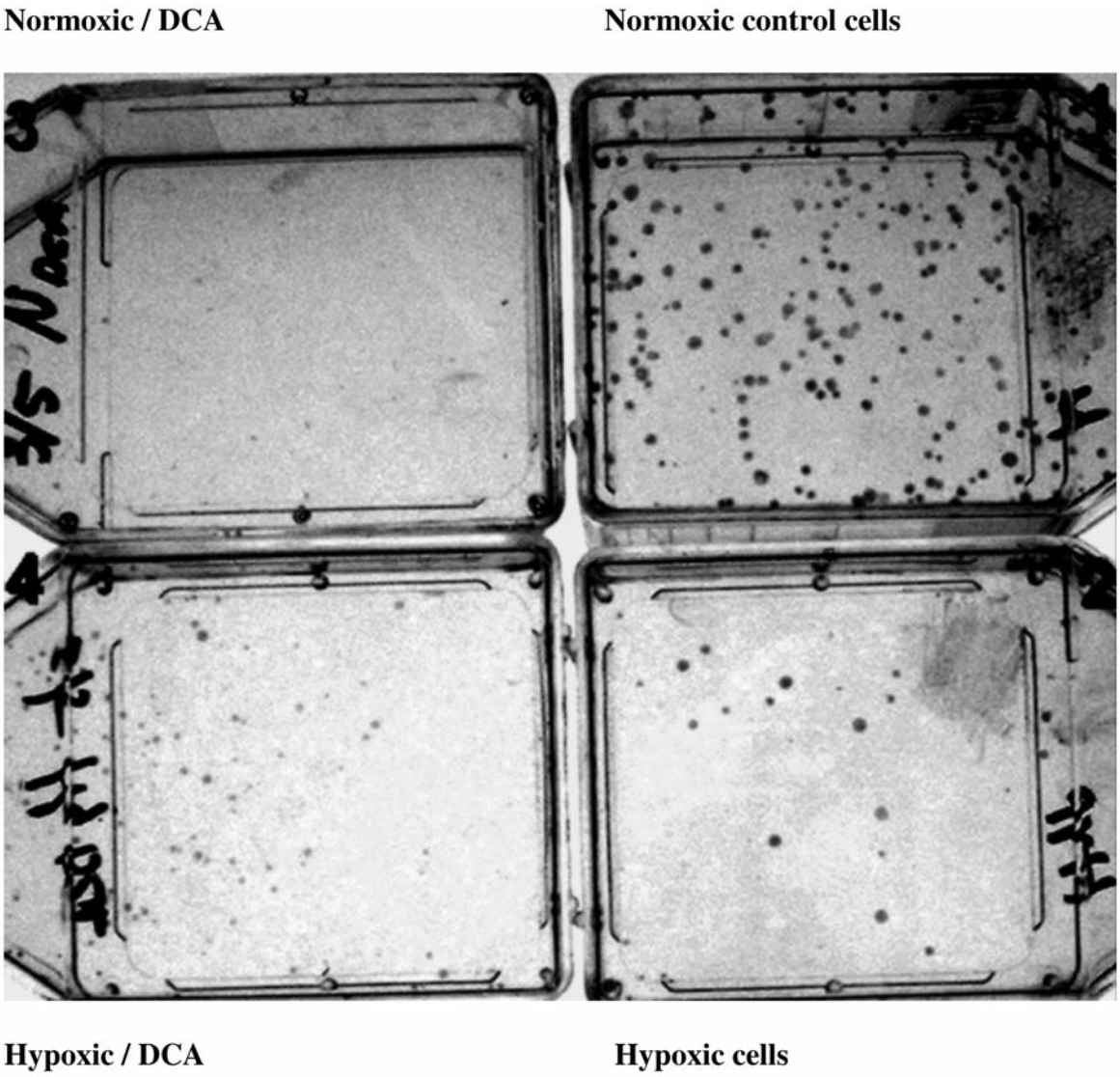

Figure 3. *Clonogenicity of hypoxic HeLa cells subsequently cultured under normoxia compared with cells continuously cultured under normoxia. Initially cells were incubated with 12.5 mM DCA under normoxia or hypoxia for 3 days, detached, washed and 500 or in other experiments 1,000 cells each incubated in 25 ml flasks under normoxia for 18-20 days. Cells were stained with methylene blue, washed and colonies containing 25 or more cells counted.*

normoxic (with 5% CO_2) conditions. In many mitogen-activated cell lines, proliferation is accompanied by increased cytoplasmic pH: in other lines, it occurs without marked change in pH (38-41). Increasing cytoplasmic pH by itself need not initiate DNA synthesis, and generally an alkaline cytosol seems to have a facultative role in DNA synthesis. In many cell lines, apoptosis and possibly related forms of cell death (17, 42) are associated with reduced cell pH, although exceptions are noted. Maintenance or increase in cytosol pH by DCA or for other reasons should blunt this response. It is also likely that the net effect of countervailing influences on apoptosis differs among cell types and experimental circumstances.

Effects of DCA on a number of metabolic features associated with normal (non-malignantly transformed) cells and cancer cells have occurred at concentrations from 0.5 to 5 mM (6) and to 10 mM (23), but the results cited above required the higher 12.5 mM DCA concentration for the most reliable inhibition of proliferation. Presumably their selective effects noted with particular malignantly transformed cells (6) are most prominent in cells with dominant and unusually active Kv1.5 ion channels. In that study, DCA increased the activity of Kv1.5 channels and apoptosis followed by a defined NFAT-dependent pathway. Interestingly, in AsO_3-induced HeLa cell death, potassium channel activity increased

and cell death followed; both were partially blocked by voltage-dependent K^+ channel inhibitors such as 4-aminopyridine (43). Induction of apoptosis by DCA in non-transformed pulmonary artery cells from animals expressing Kv1.5 and Kv2.1 voltage-dependent potassium channels and subject to pulmonary hypertension has been reported (33, 44). Numerous voltage-dependent potassium channels are described (45), but their status, especially voltage-dependent potassium channels in HeLa and PANC-1 cells was not discussed. Cellular selectivity is likely in large part determined by the presence and activity or absence of specific potassium channels as they are integrated with particular signal transduction pathways (46). These and probably other as yet unidentified factors may contribute to differences in response of specific non-malignant and cancer cells.

The effect of DCA on ATP formation correlated with increased cell numbers of normoxic and hypoxic cells measured with the MTS assay, provided they were in cycle. Once proliferation ceased and a monolayer was established, fluorescence no longer correlated as closely with cell numbers.

In the majority of experiments, there were more normoxic colonies than those of cells first cultured under hypoxia and then transferred to normoxia. The colony counts of hypoxic cells cultured with 12.5 mM DCA generally exceeded those of normoxic DCA cells when culture was continued under normoxic conditions. Due to the difficulty in accurately counting cells, some variation was encountered.

Several recent studies are especially informative for understanding the relation between normoxic and hypoxic cancer cells (47-50). It has been suggested that mutations in metabolic enzymes could serve as oncogenes or tumor suppressors (51). Whether an enforced imposition of increased cell pH could contribute to malignant transformation (52) does not seem to be established but evidence for direct participation of cytosol alkalinization in oncogenesis has been presented (53). Recent work with carbonic anhydrase IX and XII supports such a possibility (47). In these studies, induction of carbonic anhydrase (CA) IX and XII stimulated tumor growth by increasing extracellular acidification while increasing intracellular alkalosis. *In vivo*, inhibition of CAIX and/or CAXII resulted in marked reduction in xenograft volume, maximum when both were inhibited together. The ability of DCA to raise intracellular pH (6) as a potential facultative stimulus for promoting proliferation provides additional support for the importance of pH in hypoxic cell survival, as does our work, provided we can demonstrate that medium pH reflects intracellular pH (work in progress).

Recently, it was shown that oxygenated cancer cells expressing the monocarboxylate transporter 1 (*MCT1*, SLC 16A1 solute carrier family 16, member 1, monocarboxylic acid transporter) gene, required for the cellular uptake of lactate, preferentially used lactate for metabolic energy, which was provided by hypoxic cells (48, 49). When the uptake of lactate was blocked by the small molecular weight inhibitor, α-cyano-4-hydroxycinnamate (CHC), the growth both *in vitro* and *in vitro* of several cancer cell models was inhibited as oxygenated cells switched from lactate to glucose. This resulted in depleting hypoxic cells of glucose and rendered them sensitive to radiation. Hypoxic cells are considered to generate progeny expressing properties such as increased tumor vascularity, resistance to therapies, the epithelial to mesenchyme transition *etc*., all contributing to the formation of metastatic cancer. In another important study, preventing cellular hypoxia by expressing myoglobin in A549 human lung cancer cells reduced the ability of xenografts to proliferate, their blood vessel density declined, cells differentiated and metastases were infrequent (50).

It is of interest that in yeast, maximum DNA synthesis occurs under reducing conditions (20). If under certain circumstances of hypoxia the *in vitro* differences related to DCA were retained *in vivo*, especially if accompanied by a rise in cellular pH due to agents such as DCA or for other reasons (43), proliferation and survival of a developing subset of hypoxic cells could be promoted. To the extent this occurred, clonal evolution of hypoxic cells toward less regulated proliferation and increased expression of properties promoting metastasis could be favored.

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