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The Effect of Normoxia and Hypoxia on a Prostate (PC-3) CD44/CD41 Cell Side Fraction

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Abstract. *It has been reported that human prostate-derived PC-3 cells that are CD44- and CD 41 (a2 B1)-positive are enriched in cancer stem cells. This study compared the effect of PC-3 cell proliferation under normoxia or hypoxia on the initial and subsequent expression of this doubly-labeled side-fraction. Despite the numerical advantage of attached normoxic cells, 48 h of culture under nitrogen, an environment containing minimal oxygen and CO_2 resulting in an elevated pH of the medium, was associated with a higher percentage, absolute and relative number of doubly-labeled (DL) hypoxic compared to normoxic cells. At 24 h, the reverse was found. When the pH was controlled with the use of 95% nitrogen and 5% carbon dioxide, the percentage and number of normoxic DL cells exceeded hypoxic ones at both 24 and 48 h. At 24 h, 2-deoxy-L-glucose or sodium arsenate reduced normoxic DL cell numbers more than hypoxic ones. The interplay between hypoxia, increased medium pH and the effect of inhibitors as they might influence therapy are considered.*

Many solid types of cancer are believed to originate from a single malignantly transformed tissue stem cell by one and probably a limited number of additional, somewhat cell-specific somatic mutations, concealed by numerous secondary events (1-4). The number of putative cancer stem cells (CSC) associated with murine and human cancer has varied from under 1% to upwards of 25 to 30%, depending upon the system studied and the means used to identify them (5).

Several reviews have focused on putative prostate cancer stem cells and the criteria used to identify them (6-10). Putative human prostate cancer stem cells have been associated with CD 133 (prominin), CD 44 and α2β1 integrin expression, high mRNA levels of OCT3/4, BMI 1, but lack of prostate specific antigen and androgen receptor. Human teleomerase reverse transcriptase-generated human prostate cell lines capable of initiating tumors were found to be AR^-, $P63^-$ and expressed CD44, nestin, Oct-4, Nanog, Sox 2 and c-kit (11).

Based on earlier studies of keratinocytes (12), distinction between PC-3 prostate cancer stem cell holo-, mero- and paraclones, each exhibiting different morphological and functional properties, have been demonstrated (13). In PC-3 cells, the highest concentrations of CD44, α2β1 (CD41) and β-catenin were observed in holoclones, with diminishing amounts present in mero- and paraclones. Holoclones derived from single cells were capable of culture for up to six months, while the antigenic markers in para- and meroclones decreased over time and cells died much earlier. Holoclones from cultured PC-3 cells or from holoclone-initiated tumors were able to be serially transplanted and initiated tumors containing the three types of clones (13). Others found the three categories of potential cancer stem cell clones in five other prostate cell lines including DU145 and LnCaP, except that holoclones were not observed in their PC-3 cell line (14).

Schmaltz *et al.* demonstrated that preventing a fall in medium pH due to acidosis in hypoxic cells circumvented apoptosis and increased their numbers (15). With increased medium pH, the expected normal block at the G_0/G_1 checkpoint in hypoxic non-transformed cells was overridden in their malignant counterparts. Tumor cell viability and proliferation exceeded that of their normoxic controls. P53 expression in transformed, non-acidotic hypoxic cells was down-regulated, while if acidosis was allowed to develop, P53 was up-regulated. It is of interest that PC-3 cells lack P53 expression (16).

In addition, based on an earlier report that hematopoietic stem cells were present in regions of hypoxia (17), that hypoxia increased a tumorogenic and invasive side-population of a variety of cultured human cancer cell lines (18), and the question of a possible effect of a more alkaline ‘microenvironment’ on hypoxic cell proliferation and survival (19, 20), this study compared the effect of hypoxia and an alkaline environment on the content,

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Table I. *Final cell numbers, percentage CD44 + CD41 double-positive members and the calculated percentage of doubly-labeled cells. The same number of cells was cultured for 48 (or 24) h under normoxia (N) or hypoxia (H) and equal volumes of detached cells compared by flow cytometry for the parameters indicated. In expt. #4, cells were detached after 24 or 48 h of culture for comparison. Results of the third (24 h) experiment are presented in Figure 1.*

	DL	%	DL/10^5	Cell*	%DL	DL*	DL/10^5
Expt.1^{48H}							
Normoxic	119/15000	0.79	793	222	0.79	1754	790
Hypoxic	201/15000	1.34	1340	168	1.34	2251	1340 (1.7)
Expt.2^{48H}							
Input	46/15000	0.31	306	716	0.31	2220	310
N	16/15000	0.11	106	2.1×10^6	0.11	2310	1100 (0.35)
H	84/15000	0.56	560	800	0.56	4480	5600 (1.8)
Expt.3^{24H}							
N	1833/14774	12.4	12400	2.68×10^6	12.4	332320	12400
H	790/13586	5.8	5810	2.56×10^6	5.8	148480	5800 (0.48)
Expt.4$^{24/48}$							
24 H							
N	343/42924	0.80	799	330	0.8	2640	800 (13.3)
H	290/41186	0.70	704	312	0.7	2184	700 (0.9, 1.17)
48 H							
N	42/34495	0.12	122	646	0.12	775	120 (2.0)
H	67/26211	0.26	256	426	0.26	1108	260 (2.2, 4.3)
Expt.5+6 under N_2/5%CO_2							
Expt.5							
Input	121/15066	0.80	803	1.16×10^6	0.8	9280	800
24 H							
N	1003/12866	7.8	7796	594	7.8	4630	7800 (9.75)
H	638/9824	6.5	6494	300	6.5	19500	6500 (0.83, 8.1)
48 H							
N	81/12527	0.65	646	384	0.65	2496	650 (0.81)
H	36/12590	0.29	285	326	0.29	945	290 (0.36)
Expt.6							
Input	158/15000	1.05	1053	629	1.05	7270	1050
24 H							
N	1122/11692	9.6	9596	180	9.6	17280	9600 (9.1)
H	313/11342	2.8	2760	150	2.8	4200	2800 (2.7)
48 H							
N	145/17334	0.84	840	380	0.84	3192	840 (0.80
H	65/9307	0.70	698	202	0.70	1414	700 (0.67)

DL: Number of CD44 + CD41/cells counted; % DL: number of DL/10^5 cells; Input: initial number of cells seeded; cell number*: $\times10^3$ detached after 24 or 48 h cells; % DL assumed same; number of DL*: detached cells; number of DL/10^5: detached cells/10^5. Relative DL density is per 10^5cells; absolute DL concentration uses the number of detached cells × the % DL from flow cytometry. Ratios of hypoxic DL to normoxic or input DL cells are shown in parentheses.

behavior and response of a doubly-labeled (DL) PC-3 CD44/$\alpha2\beta1$ integrin 'side fraction', considered to be enriched in PC-3 cancer stem cells and reported to contain holo-, mero- and paraclones (13).

Materials and Methods

Cell culture. PC-3 prostate cancer cells from the American Type Culture Collection (Bethesda, MD, USA) were cultured in RPMI containing 10% FBS, 50 U/ml penicillin, 0.05 mg/ml streptomycin, 25 mM HEPES and GlutaMax^R (Gibco, Carlsbad, CA, USA) for the times indicated, 24 and 48 h. Quiescent cells at time '0' had generally been sub-cultured for three or four days before use, while actively dividing cells were split the day before detachment and re-feeding.

Procedure used to achieve 'hypoxia'. Normoxia' represented cells cultured under standard conditions of an ambient atmosphere supplemented with 5% carbon dioxide. A volume of 5 ml of water was used to maintain humidity of the hypoxic cells while phenol red in the RPMI reflected the final pH of the medium. In some experiments, in order to maintain the pH nearer to 7.4, nitrogen was supplemented with 5% CO_2. Hypoxia within a bell jar was achieved by flushing it with nitrogen for one minute, when the flow of gas was reduced while gradually closing the cover of the jar. It is likely that some variation in the degree of hypoxia was present from one experiment to another, however the final pH of the media after 48 h of incubation was 7.8 compared to 7.2-7.4 for normoxic samples. Occasionally, if closure of the cover and reduction in nitrogen flow were not synchronized properly, a pH in excess of 7.8 was obtained. The sealed bell jar was kept at 37˚C in a 'normoxic' incubator for

the times indicated and the resulting hypoxia was confirmed by the rise in pH during the incubation.

Residual oxygen in predominantly nitrogen-containing gases has been estimated to be of the order of 10 ppm. Estimates of residual oxygen and carbon dioxide in the medium are available in (20) but no attempt to deplete the RPMI of either gas was intended.

Immunologic studies. Quiescent cells that had not been sub-cultured for three to six days were detached with trypsin-EDTA, washed once with Dulbecco's PBS, their concentration measured with trypan, cells distributed in flasks and cultured under normoxia or hypoxia for 48 or 24 h. Subsequently, cells were detached, washed twice at 4˚C with Dulbecco's PBS, re-suspended in buffer containing 10 mg BSA/ml, their density determined and antibodies and isotypic controls added to cells as follows. (i) FITC - CD44 + PE - CD41 isotypic control); (ii) FITC - isotype + PE - CD 41; (iii) CD44 + CD41; (iv) CD 44 isotype + CD41 isotype. The double isotypic control (iv) was subsequently omitted. Samples were kept for 1 h at 4˚C , washed twice with cold PBS/BSA and re-suspended in PBS at approximately 10^6 cells/ml. Samples were submitted to the Resources Research Center Flow Cytometry Lab. at the University of Illinois Medical School in Chicago for analysis.

Cell proliferation studies. A commercial MTS-related assay (Promega, Milwaukee, WI, USA) was used to estimate cell viability and imputed proliferation. A reagent blank was included in the final calculation of optical density at 490 nm.

Inhibitor studies. *N-Tert*-butyl phenylnitrone (NTBN), a free radical spin trap, 2-deoxyglucose, an inhibitor of glycolysis, and sodium arsenate, an uncoupler of oxidative phosphorylation, were employed at the concentrations cited in Tables II and III. Cells were cultured under the conditions described and the results of treatment estimated by the MTS assay.

pH determination. Phenol red and litmus paper were used to determine major differences in pH of normoxic and hypoxic samples. Attempts to quickly measure pH with a pH meter were compromised by the rapid rise in the ambient atmosphere.

Materials. Unless otherwise stated, chemicals and reagents were obtained from Sigma (St. Louis, MO, USA). RPMI with GlutaMax, with or without phenol red was obtained from Gibco, MTS Cell Titer 96 from Promega Corp (Milwaukee, WI, USA), FTIC-CD44 and an isotypic control from BD Biosciences (Chicago, IL, USA) and PE-CD 41 and its isotypic control from Abcam (Cambridge, MA). CD 41 has also been designated as BAK (platelet-specific), ITGAB, ITGA2B or integrin alpha 2B.

Table II. *Effect of selected inhibitors on proliferation of PC-3 cells for 48 hours.*

	O.D. 490 nm	
	Normoxic	Hypoxic
Control	0.22±0.02	0.27±0.01
2-DG	0.12±0.04	0.11±0.02
NTBN	0.05±<0.01	0.06±0.05
DG+NTBN	0.05±0.02	0.06±0.05
AS	0.05±0.01	0.03±0.03
MS	0.18±0.04	0.29±0.05
MS + AS	0.01±<0.01	0.00
MS +AS +DG	0.01±<0.01	0.00

Ninety-six well plates were used with final concentrations of 2-DG: 15 mM; NTBN: 15 mM; sodium arsenate (AS): 1.7 mM and L-methionine sulfoxamine (MS), an inhibitor of glutaminase, 3.3 mM. N=3 to 5; reagent blanks were subtracted.

Results

Immunologic studies. Figure 1 depicts a representative example of data from Table I, expt. 3. In the first experiment, the percentage of DL PC-3 cells was measured and the absolute number (detached cell number × %DL) and relative number of DL cells (%DL $\times 10^5$ cells) that had been released by trypsin after 48 h of culture under normoxia or hypoxia was calculated (Table I, expt. 1). As was expected, hypoxia limited their proliferation. The previously attached 'hypoxic' cell fraction contained 1.34% DL cells, compared to 0.79% in the detached normoxic cell fraction. When these percentages of DL cells were applied to the detached cells in each fraction, approximately twice the number of DL cells was present in the hypoxic sample, despite their lower number. The next study included the DL cells in the input fraction of cells (Table I, expt. 2). There was a greater percentage and absolute number of DL cells in the detached hypoxic DL cell fraction than normoxic one. The relative number ($DL/10^5$) of hypoxic cells ($560/10^5$) was less due to the smaller number of detached hypoxic cells. The fold-differences of hypoxic to normoxic cells was 1.7 (expt. 1) and 1.8 (expt. 2). One interesting feature of the second experiment was the somewhat greater number of hypoxic over input cells, consistent with proliferation of DL hypoxic cells in excess of the input DL cells.

To avoid missing a possible early increased replication of attached DL cells, they were examined after 24 h (Figure 1, Table I, expt. 3). More DL cells were present in normoxic than hypoxic samples. The number of DL cells in the input samples was not measured. The fourth experiment combined these issues (Table I, expt. 4). DL fractions after 24 and 48 h of culture were compared. At 24 h, normoxic DL cells were more numerous than hypoxic DL cells ($800/10^5$ compared with $700/10^5$). After another 24 h of culture, these numbers markedly declined, with a reversal of the relative numbers of hypoxic DL cells ($260/10^5$) compared with normoxic cells ($120/10^5$).

Effect of a 'physiologic' pH of 7.4 on DL cell number. To study the effect of pH on these results normoxic and hypoxic cells cultured in 95% N_2, 5% CO_2 which kept the pH of the medium at 7.3-7.4 were compared (Table I, expt. 5 and 6).

Table III. *The effect of several inhibitors on the CD44 + CD41-labeled cell fraction. Cells were incubated under nitrogen with 2-deoxyglucose (2-DG) or sodium arsenate (AS) for 24 h, cells detached, washed, labeled with antibodies, incubated at 4˚C, washed and examined by flow cytometry as described previously. Input cell numbers were 1.3×10^6 and 4.6×10^5 and concentrations of 2-DG of 15 mM and 30 mM and of AS of 0.72 and 1.4 mM, respectively. As before, the same percentage of DL cells was used to calculate both relative and absolute concentrations.*

	DL	%	DL/10^5	Detached	%	# DL
#1						
Control						
N	1475/15159	9.7	9370	268000	9.7	26090
H	290/14489	2.0	2000	94000	2.0	1880
2-DG						
N	890/15447	5.8	5760	232000	5.8	13456
H	2283/14006	16.3	16300	252000	16.3	41080
AS						
N	396/12848	3.1	3080	230000	3.1	7130
H	634/14554	4.4	4360	184000	4.4	8100
#2						
Control						
N	90/17682	0.51*	510	1147000	0.51	745
H	56/7796	0.72	720	78000	0.72	562
2-DG						
N	62/11482	0.54	540	100000	0.54	540
H	66/9020	0.73	730	90000	0.73	660
AS						
N	5/3580	0.14	140	36000	0.14	50
H	14/7015	0.20	200	38000	0.20	76

N: Normoxic; H: hypoxic.

At 24 h, both normoxic and hypoxic DL cells exceeded the input DL cell number by nearly 10-fold (Table I, expt. 5). At both 24 and 48 h, normoxic DL cells exceeded hypoxic ones by the factors indicated. In experiment 6, results were similar, with a greater percentage, relative and absolute numbers of DL cells in the normoxic fractions at both 24 and 48 h.

Effect of several inhibitors on PC-3 cell proliferation and the percentage of DL cells at 24 hours. First the effect of several inhibitors on the proliferation of PC-3 cells, assessed with an MTS assay, was examined (Table II). Two-deoxyglucose, NTBN, a spin trap, and sodium arsenate, a non-specific phosphatase inhibitor were effective inhibitors of proliferation, and in combination essentially halted it.

The effect of 2-DG and arsenate on normoxic or hypoxic DL cells after 24 h was determined (Table III). Control hypoxic cells exhibited a smaller (expt. 1) or unexpectedly a somewhat greater (expt. 2) percentage of DL cells than normoxic ones. However, with the additional stress of 2-DG or arsenate, the percentage and absolute numbers of hypoxic DL cells exceeded their inhibited normoxic controls. The second experiment, employing increased concentrations of inhibitors and fewer input cells, yielded qualitatively similar results to that with the inhibited samples.

Discussion

After 48 h of culture under nitrogen containing minimal residual oxygen and carbon dioxide and a medium pH of 7.8, the percentage, absolute and relative number of hypoxic DL PC-3 cells exceeded those of normoxic DL cells, even though non-DL normoxic cells were present in greater numbers. At 24 h of culture, the percentage and the absolute and relative normoxic cell numbers exceeded hypoxic values. As noted, under nitrogen, the final pH of the medium was 7.8. When the same comparisons were made of cells cultured under nitrogen-5% CO_2, which normalized the pH, the percentage, absolute and relative numbers of normoxic DL cells exceeded the hypoxic values at both 24 and 48 h. In experiments 2, 4, 5 and 6, the absolute and relative numbers of DL cells exceeded the input DL cell number, implying hypoxic DL cells proliferated. Under the conditions employed, the number of total detached normoxic cells consistently exceeded that of hypoxic cells, although in some experiments the differences were not marked.

Taken altogether, these results support the argument that during 48 h of culture, normoxic or hypoxic DL PC3 cells, exposed to the proliferative stimuli of trypsinazation and fresh medium, replicate approximately in proportion to the input number of cells and to a lesser extent to the percentage

of DL cells. It was interesting that a majority of both the normoxic and hypoxic input cells did not attach and proliferate. Presumably this included 'differentiated' cells no longer able to replicate. Since more hypoxic DL cells were present after 48 h in a nitrogen environment, compared with culture under nitrogen /5%CO_2, one possibility is that a more alkaline medium (15, 19-21) is supportive of DL hypoxic cellular survival.

It is believed that much of the variability encountered was due to the use of a bell jar with differences from experiment to experiment between shutting off the nitrogen flow while simultaneously closing the lid of the jar. It would be interesting to examine the effect of hypoxia from perhaps 2% oxygen to its virtual absence on replication of cells of interest, but more specialized equipment would be required.

After 48 h, the numbers of DL cells in normoxic and hypoxic cultures were considerably reduced. One assumption, in addition to the belief that these DL cells were viable, is that DL cells originally present at 24 h had replicated, but subsequently lost their DL identification and merged with the pool of cells, both those that initially attached and others supplemented by replication. The PC-3 cell cycle is approximately 24 h. Dilution among proliferating 'transit amplifying cells' with loss of immunologic markers after proliferation of the 'input' DL cells may account for the steep decline as DL cells between 24 and 48 h. They enter the 'transit amplifying pool' of replicating cells in insufficient numbers to account for the majority of cellular proliferation. Loss from DL cells from apoptosis seems unlikely as a more alkaline medium militates against apoptosis (15).

Presumably what is occurring is a form of a 'sum over time' in which the prior history of the culture determines the events observed. The problem resembles that confronting statisticians estimating mortality rates of populations. Previously, an aliquot from a PC-3 cell culture with an unknown number of cells in or out of cycle was selected for archiving in American Tissue Culture Collection. Subsequently these cells were cultured and allowed (in this study) to become quiescent due to contact inhibition and medium exhaustion. This quiescent culture included some DL cells, greater number of transit amplifying cells probably including mero- and paraclones distributed throughout the cell cycle, others blocked at the G_1/S boundary and additional numbers of 'differentiating' cells, gradually less able to proliferate (8, 13). In addition to whatever 'programs' mandate further development of PC-3 cells, some of these groups are likely undergoing genetic and epigenetic changes, altered immunologic markers, declining ability to proliferate, gradually 'differentiating' and/or undergoing various forms of cell death.

Following detachment and exposure to fresh medium, both normoxic and hypoxic cells, out or in cycle, entered or resumed proliferation as the aforementioned events continued. The total increase in cell numbers was not due directly to the limited numbers of DL cells but was strongly dependent upon the transit amplifying cells ($CD44^+$, $CD41^-$ and others among them) generated during the cultures' prior history and continued as part of the events then in progress. This is seen by comparison of the percentage and number of DL cells in the input culture compared with those present after 24 h.

It can be argued that the increased number of DL cells in 48 h hypoxic compared to normoxic cultures was due to the inability of hypoxic DL cells to continue to replicate and progress further into the transit amplifying pool. Recruitment during 24 h from some unidentified DL precursor pool might be invoked (22). According to this view, either additional DL antigen-labeled cells are 'uncovered' from some 'reserve' of stem cell precursors and/or other cells are in some other way 'recruited' into this DL pool. Of course, other presently unidentified but immunologically distinct potential stem cells might also be present. It seems that the initial increase in DL cells in both hypoxic and normoxic 24 h samples represent true DL cell replication with subsequent 'flow through' of proliferating DL and other PC-3 cells. Passage into and through the cell cycle becomes progressively limited, especially for hypoxic cells. This increases the number of residual hypoxic DL cells with their reduced maturation as transit amplifying cells under the increasingly oxygen and CO_2-depleted medium of nitrogen-induced hypoxia. Determining the cell cycle distribution of hypoxic DL cells should settle this point (work in progress).

2-Deoxy-L-glucose strongly inhibited the proliferation and survival of normoxic or hypoxic PC-3 cells studied in 96-well plates. The same concentrations of inhibitors had less effect on cells cultured in 75 cm^2 flasks. The effect of inhibitors on hypoxic DL cell survival was similar to that seen by others (1-3), based on studies with chemotherapeutic agents or ionizing radiation; that more hypoxic stem (also known as DL) cells survived than normoxic ones. The calculated number and percentage of inhibited hypoxic cells was 1.13 to 3 times and 1.35 to 2.8 times, respectively, those of inhibited normoxic cells, depending upon the inhibitor and the experiment.

In the present experiments, normoxic PC-3 cell numbers exceeded hypoxic numbers. In studies with HeLa cells, hypoxic conditions were achieved in which initially, hypoxic cell numbers were greater than normoxic numbers as judged by an MTS assay of cells cultured in 96-well plates (20). Under culture conditions using flasks in which continued expansion of the monolayer was able to occur, normoxic cell numbers subsequently exceeded hypoxic ones. The MTS assay measures dehydrogenases that are able to convert the substrate to a colored compound, and this is taken to reflect cell viability and cell number. Performing the assay with hypoxic cells at a higher pH might give rise to artifacts (23). However, direct counting of cells in

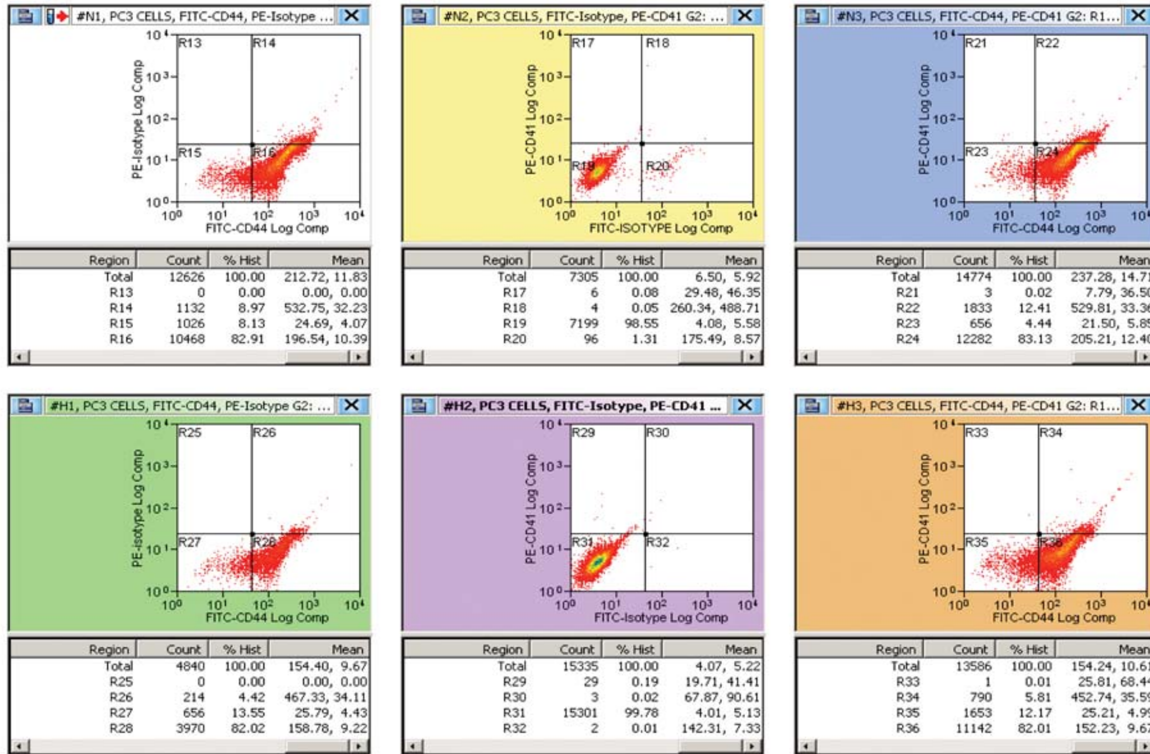

Figure 1. *Flow cytometric study of an identical number of PC-3 cells cultured for 24 h under normoxia or hypoxia and examined for double-staining with CD 44 and CD 41 (expt. 3 of Table I). Upper left and center panels, normoxic cells plus antibody with/without isotypic controls. Right hand panel, dual antibodies normoxic. Lower left and center panels, hypoxic cells with isotypic controls. Right lower panel, hypoxic cells with dual antibodies. Each individual panel: right upper quadrant, DL cells; lower right and upper left quadrants, P44 (or P41) antibody and P41 or P44 isotypic control; lower left quadrant, unlabeled, isotypes or dead cells.*

different culture vessels and theuse of an ATP assay to reflect cell numbers supported the interpretation that, under yet to be defined conditions, hypoxia in the presence of a limited but undetermined concentration of oxygen and CO_2, combined with medium of an increased pH, provided an initial stimulus to hypoxic cell proliferation. Using 96-well plates, once contact inhibition is achieved or the medium is exhausted, neither PC-3 nor HeLa cells overgrow the initial monolayer. HeLa cells express P53 while PC-3 cells are *P53*^{null} (16). This difference may complicate these comparisons. In malignantly transformed, non-acidotic hypoxic cells, P53 was down-regulated and the expected G_0/G_1 checkpoint overridden (15). Permitting acidosis to develop had the opposite effect and promoted apoptosis. Variations among HeLa cell preparations in the degree of hypoxia and the concentration of residual oxygen probably contributed to the results in experiments in which hypoxic cells did not initially exceed the normoxic controls. A number of reports confirm the occurrence of hypoxic proliferative stimuli under various experimental circumstances (some were cited in (20)).

None of this helps to elucidate whether cancer as represented by PC-3 cell proliferation supports a stochastic clonal selection model or the cancer stem cell model. In the stochastic model, essentially all the progeny of the Ur-cancer stem cells are potentially endowed with tumorigenic proliferative capacity. Sub-clones develop due to the influence of the microenvironment, supplemented by random or possibly less than random genetic and epigenetic changes occurring over time. The hierarchic cancer stem cell model envisions a more limited number of replicating cancer stem cells undergoing self-renewing, asymmetric stem cell division This generates a continuing source of cancer stem cells, some kept in reserve. Proliferating daughter cells are fated to undergo the developmental events described above.

The ability of a single transformed solid or hematopoietic cancer cell to duplicate the original cancer in an immunologically deficient mouse does not exclude other related but still distinct cancer stem cells from the same source also being able to do so. Examples of cancer cells with different phenotypes originating from a single

malignantly transformed clone have been reported (24). Evidence is available that transformed progenitor cells, probably including transit amplifying cells can revert to cells with stem cell properties (25, 26). As procedures are refined the number of cancer stem cells detected has increased. Modifications including the length of the study, type of immunocompromised mice and the use of Matrigel[R] increased the detection of melanoma stem cells to one in four cells studied (27). In a companion study of tumorigenic and non-tumorigenic melanoma cells involving 50 markers, no evidence of a hierarchical organization was seen. In a comparison of non-malignant cells, high-order gene expression was found in adult stem cells that differed from that of embryonic and differentiated cells, consistent with expression of similar stem cell gene subsets (28).

If transformed stem cell variants besides the candidate CD44 + CD41-marked cells are included among the initial input of cells (4, 21), consistent with a clonal evolution stochastic model, is responsible for PC-3 tumorigenesis, simply targeting CD44 + CD41 cells should be inadequate. If a strict stem cell model is present, and a unique cancer stem cell or stem cell-like transition from CD 44 + CD41 holo- to mero- and paraclone to transit amplifying, replication-capable side fraction-derived cells could be reduced by therapy, a longer tumor latency could result.

Provisionally, the percentage of nitrogen-hypoxic *versus* normoxic DL cells and their absolute and relative numbers favor the hypoxic cells but the data is limited. More hypoxic DL than normoxic DL cells remained after the stress from 2-deoxy-L-glucose or arsenate. Whether this was due to their inherent properties or to the lack of entrance into the transit amplifying population is uncertain but the conclusion is consistent with reports of others.

If, as seems to be the case, an alkaline medium promotes cellular proliferation and survival (15, 20, 21) and to the extent that hypoxia may at times be associated with local alkalinization (29), due to enzymes such as carbonic anhydrase IX or XII that enhance local alkalinity (30) or appear around regions of necrosis, cells exposed to these conditions may develop a selective survival/growth advantage. Compared to normoxic cells, the greater percentage and number of residual (48 h) DL hypoxic cells provides increased opportunity for genetic or epigenetic changes. Hypoxic cells are prone to greater genetic changes than normoxic ones (31) and some of them could be detrimental to a host.

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