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Bioenergetics of Human Cancer Cells and Normal Cells During Proliferation and Differentiation

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Abstract

Cancer cells are known to have different metabolic properties than normal cells, particularly their tendency to undergo glycolysis even under aerobic favoring conditions. This has created interest in how mitochondrial function in tumor cells may differ from that in normal cells. Using human malignant cells (SW-620, PC-3, HT-1080, SK-MEL, HL-60, K-562 and MOLT-3), human fibroblast (CCL-153) and human T Cells, we investigated three key parameters that have been typically used to describe mitochondrial function: cellular ATP production, mitochondrial potential and cellular cardiolipin levels. On average, tumor cancer cells had more ATP production and greater mitochondrial potentials. For example, ATP levels in malignant cells ranged from 20 to 69 µmole/10^6 cells, with a cancer cell average of 40 ± 18 µmole/10^6 cells. For normal cells, the ATP level range went from 9 to 24 µmole/10^6 cells, for an average of 15 ± 11 µmole/10^6 cells. Mitochondrial potentials tended to be three times higher in cancer cells, perhaps because overall mitochondrial mass (as measured by relative cardiolipin levels) were twice as high in cancer cells. Higher mitochondrial masses are consistent with proliferation. Proliferating cells in general showed higher mitochondrial function compared to quiescent cells (confluent monolayers), and HL-60 cells showed reductions in all three mitochondrial parameters measured here when the cells were exposed to the differentiating agent TPA. The effects of ATP production inhibitors CCCP and oligomycin on mitochondrial function in normal and cancer cells were also compared. In general, in these experiments, cancer cell mitochondrial inhibition with these agents produced a decrease ATP levels by 30-40% while in normal cells ATP production was reduced by 60%. These results provide evidence of a mitochondrial dysfunction in cancer cells. Cancer cells appear to better withstand interference with ATP synthesis in mitochondria since they rely mainly on glycolysis as an energy producing mechanism.

Introduction

Research into the energy metabolism of cancer cells began in the early 20th century with Otto Warburg, who observed that tumor tissues appear defective in respiration and have abnormally high rates of aerobic glycolysis [1]. This led Warburg to propose that cancer arouse as a result of mitochondrial injury [2]. Since then several cancer cell metabolism and mitochondrial function has been subject to extensive study. Two of the most well-known and accepted features of tumor cell metabolism are the "Crabtree effect" [3] and the "Pasteur effect" [4]. The former refers to inhibition of cancer cell respiration by elevated glucose concentrations, while the latter refers to inhibition of glycolysis by elevated oxygen concentration. Presumably, the Crabtree effect arises due to competition between glycolysis and oxidative phosphorylation for Pi and ADP [5]. It has also been observed in some cancer cells that the consumption rate of one nutrient (oxygen or glucose) increases when concentration of the other nutrient is reduced, suggesting an ability of cancer cells to adjust their metabolism based on micro-environment [6]. These observations show that cancer has a very relevant metabolic component.

A variety of abnormalities in cancer cell mitochondrial structure and function have been reported [7-27]. These peculiarities in glucose metabolism may be linked to differences between the mitochondria of cancer cells and those of normal cells [15-27]. These include increases in, and alteration of, mitochondrial DNA [8, 10-14,20-21], elevation of hexokinase production [15], lysis of cristae structures [23-26] and altered mitochondrial protein and lipid content [24-26]. Interestingly, genetic alterations in cancer cells, such as deregulation of the PI3K/Akt pathway, or imbalances in activity of c-MYC, HIF, or p53, can alter glucose and amino acid metabolism [7,28]. In

addition, cancer cells have abnormal content and composition of cardiolipin [29-33], a key mitochondrial lipid that is necessary for proper cell respiration. Cardiolipin normally resides in the inner membrane of mitochondria, where it plays a role in chemiosmosis. In addition, cardiolipin can move to the outer mitochondrial membrane to trigger apoptosis. The "defect" in mitochondrial respiration reported by Warburg may be related to cardiolipin defects in tumor cells [34]. A generalized increase in anabolism characterizes nearly all cancer types [35-36], perhaps indicating consumption of metabolic intermediates toward anabolic reactions, and concomitantly less conversion of pyruvate to oxaloacetate, leaning toward an augmented formation of lactic acid thus indicating an increase dependency on glycolysis as energy mechanism.

In the present study, we examine differences between cancer cells and normal cells in three parameters related to mitochondria: ATP production, cardiolipin concentration, and mitochondrial potentials. Moreover, we examine how, in cancer cells and normal cells, these parameters are affected by ATP synthesis inhibitors and, in case of one cancer cell type, chemically induced differentiation. Our results support the idea that mitochondria in cancer cells rely more in glycolysis as their main energy production mechanism.

Methods

Cells

Human cancer cell lines used in this study include HL-60 cells (acute promyelocytic leukemia), K-562 cells (chronic myelogenic leukemia, lymphoblast), MOLT-3 (acute lymphoblastic leukemia, T cells, human), SK-MEL (skin melanoma), PC-3 (prostate carcinoma), HT-1080 (connective tissue, fibrosarcoma), CRL-1977 (uterine sarcoma), and SW-620 (colon adenocarsinoma). Normal human cell lines used in this study include CCD18-lu (lung fibroblasts) and CCL-153 (lung fibroblast). All cell lines were obtained from ATCC (Manassas, VA). In addition, T-lymphocytes were obtained from peripheral blood by incubation in RosetteSep™ antibody cocktail (StemCell Technologies). RosetteSep™ crosslinks unwanted cells to red blood cells, forming immune-rosettes. These immune-rosettes pellet during centrifugation, leaving untouched, highly purified target cells at the interface between the plasma and the density gradient medium.

ATP Measurements

Cellular ATP levels were measured using the CellTiter-GLO Luminescent Cell Viability Assay Kit (Promega, Madison WI). This assay generates a luciferase reaction that is proportional the amount of ATP present within the cells. ATP concentration versus luminescence is linear between zero and 1.5 Million cells ($r = 0.90$), allowing ATP levels (µmoles) per 10^6 cells to be determined from luminescence signals using standard curves generated with pure ATP (Sigma, St. Louis MO).

Mitochondrial Potential Measurements

Mitochondrial potential was measured based on fluorescence intensity of JC-1 (Cayman Scientific, Ann Arbor MI), a dye that exhibits potential-dependent accumulation in mitochondria. The dye's emission shifts from green (535 nm) to red (595 nm) in mitochondria, with the red-to-green ratio indicating mitochondrial potential. To exclude the effect of membrane emission during measurements, membrane potential was dissipated by gentle buffers which do not affect mitochondria (Promega lysis buffer, or by PBS with 0.2% Triton –X, 1mM DTT) before fluorescent spectra were measured. Cells were counted, and 5 x 10^5 cells were stained using 2.5 µg/mL JC-1. After wash and membrane lysis, emission spectra in range 500 nm to 750 nm were obtained using a SPEX fluorometer (SPEX Industries, Edison NJ). The total accumulation of dye at 595nm is proportional to the mitochondrial potential and the number of mitochondria, while the signal at 535 nm should be independent of this. Thus, we used the ratio of fluorescence at 595 nm to that at 535 nm as our measure of mitochondrial potential.

Cardiolipin Measurements

We used 10-N-nonyl acridine orange (NAO) dye as a cardiolipin probe.NAO monomers have a green fluorescence (flow cytometry channel FL-1) while dimers, formed on contact with cardiolipin, emit a red fluorescence (flow cytometry channel FL-3). NAO specifically binds to cardiolipin with a stoichiometry of 2:1. To measure cardiolipin, 0.5M cells were incubated in medium with NAO (dye concentration in range 1-12 µM). After the 30 min of incubation at 37C, 50µg/ml propidium iodide (PI) was added to stain the DNA of dead cells. Emission of NAO was measured by flow-cytometer.

Cell Differentiation

The HL-60 leukemia model allows us to examine the effects of differentiation on mitochondrial metrics. 12-O-tetradecanoylphorbol-13-acetate (TPA) induces cellular differentiation of a number of leukemia cell lines including HL-60. TPA was dissolved in DMSO. HL-60 cells were plated in 6-well plates with concentration 10^6 cells in 5ml growth medium. Cells were then treated with TPA (Sigma, St. Louis MO). Two TPA concentrations were tested, 32 nM and 64 nM. After 24hours, control and TPA treated cells were used in assays described above.

Results

Three key variables were assessed in this study, mitochondrial potential, mitochondrial mass and cellular ATP concentration. Results for several tumor and normal cell types are given in Table 1. There was some variation in values with cell type, but there was a general pattern of the cancer cells having higher ATP levels, greater mitochondrial mass levels (as determined by cardiolipin levels) and higher mitochondrial potentials. This confirms the hypothesis that cancer cell mitochondria have different properties than normal cell mitochondria, consistent

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with the differences in cancer cell metabolism described in the introduction. Some, but not all, of the variation in mitochondrial potential and ATP production can be explained by differences in mitochondrial mass, as shown in Figure 1(a). According to these data, levels of ATP show a statistically significant correlation with cardiolipin levels in normal cells (r=0.8) and relation with ATP in cancer cells with lower measured ATP for higher levels of cardiolipin. When mitochondrial potential or ATP levels are normalized with mitochondrial mass (dividing by cardiolipin), they are roughly thirty percent higher in cancer cells than in normal cells. This suggests that the larger mitochondrial mass in cancer cells may account in part for their increased ATP production and mitochondrial potentials, although it should be noted that cardiolipin levels may be an imperfect corollary to mitochondrial mass if cardiolipin concentrations vary significantly from one cell type to another. As expected, ATP production rates are highly correlated with mitochondrial potentials (Figure 1b) with higher measured ATP in normal cells in comparison with cancer cells at the same value of mitochondrial potential.

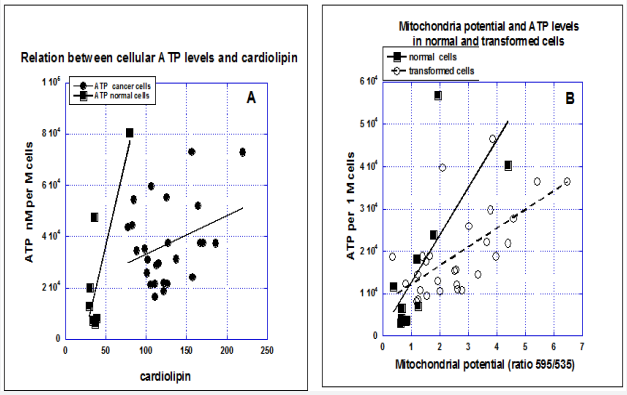

Figure 1: Relationship between (A) cellular ATP levels (μmole/10^6 cells) and cardiolipin (NAO intensity); coefficients of correlations are R=0.33 for cancer cells and R=0.82 for normal cells; (B) mitochondrial potential (ratio of emission 595nm/535nm) and ATP levels for normal and cancer cells; correlation coefficients are R=71 for normal cells and R=0.63 for cancer cells.

Table 1: Cellular ATP levels (given as μmoles per 10^6 cells), relative cardiolipin levels (given as flow cytometry peak NonylAcridine Orange emission readings), and relative mitochondrial potentials (given as ratios of JC-1 fluorescence peaks at 595 nm to those at 535 nm) for several normal cells and tumor cell lines.

Cell line	Cell type	ATP (uM/Mcells)		Cardiolipin (NAO intensity)		Mito Ratio 595/535	
		Mean	SD	Mean	SD	Mean	SD
	Normal cell lines						
T cells	Ex Vivo WBCs, healthy	8.6	3.0	35.0	4.0	0.6	0.2
WBC	white blood cells, healthy	14.1	5.0	30.4	6.1	1.4	0.4
CCD-153	fibroblast normal, lung	24.0	23.3	57.7	31.1	0.7	0.1
	Cancer cell lines						
HL-60	acute promyelocytic leukemia	28.3	3.6	137.0	20.9	3.2	1.8
J-774	mouse, tumor macrophages	44.7	10.1	145.9	26.5	4.5	1.0
K-562	myelogenic leukemia	39.5	3.1	138.6	49.4	2.9	1.1
Molt	acute lymphoblastic leukemia, T cells	20.0	2.4	118.5	7.1	2.2	1.2
PC-3	prostatic carcinoma	68.6	34.3	79.7	4.7	4.7	1.2
S180	sarcoma, mouse	38.2	24.2	115.7	13.8	4.0	0.3
SK-Mel	malignant melanoma	63.2	11.2	153.9	67.7	6.6	1.9
SW 620	colon adenocarcinoma	28.3	3.7	101.9	0.6	1.7	0.0
HT-1080	Fibrosarcoma	43.0	14.3	97.9	9.0	4.4	3.0

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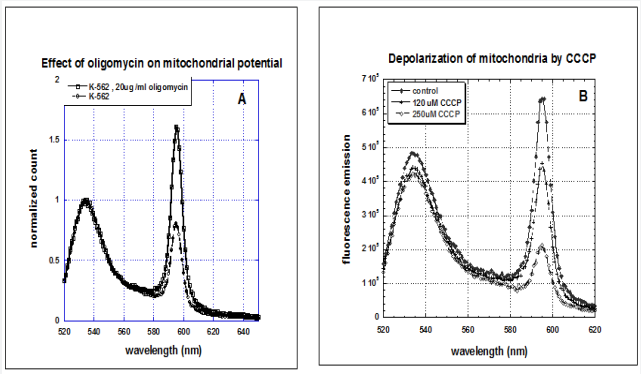

Figure 2: Fluorescence emission of JC-1 dye in K-562 cells alone or exposed to oligomycin (A) or CCCP (B).

We next examined how tumor cells and normal cells are affected by mitochondrial inhibitors oligomycin and carbonyl cyanide m-chlorophenylhydrazine (CCCP). Fluorescence emission spectra for K-562 leukemia cells loaded with JC-1 dye, as shown in Figure 2, yield two peaks, one at roughly 535 nm and another at 595 nm. Exposure of cells to oligomycin, an ATP-synthase inhibitor that increases mitochondrial potential via buildup of hydrogen ions, increases emission at 595 nm. In contrast, exposure to CCCP, an ionophore that reduces mitochondrial potential, decrease emission at 595 nm. Note that neither inhibitor prevents the electron transport chain from operating; they simply undo its potential building work (CCCP) or prevent the proton gradients generated from being used to produce ATP (oligomycin). We examined the effects of one or both inhibitors on the mitochondrial parameters described above.

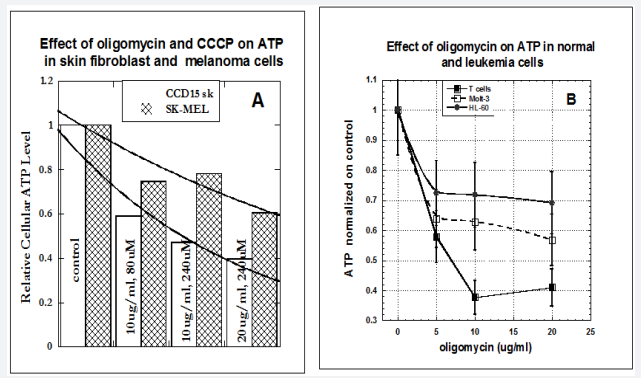

Figure 3: a) Cellular ATP levels (normalized to untreated controls) as affected by combinations of oligomycin and CCCP A) in skin derived fibroblasts (CCD-15sk) and melanoma cells (SK-Mel), B) healthy T cells and two leukemia cell lines HL-60 and MOLT.

Cellular ATP levels change as a result of inhibitor exposure for the melanoma cell line SK-Mel and the skin-derived fibroblast cell line CCD-15sk in shown in Figure 3a. The inhibitors, as expected, reduced ATP, but with there is a much greater effect on the fibroblasts (normal cells) than on the melanoma (malignant) cells. We were able to confirm this trend for a variety of tumor and normal cell types. The results for T cells and two leukemia cell lines are shown in Figure 3(b).

For T-lymphocytes, ATP levels reduced to 60% of their control levels, while the corresponding ATP level reductions for HL-60, and MOLT tumor cells were 40% of control and 30 % of control. It appears that cancer cells are able to do a better job of maintaining ATP production in the midst of mitochondrial damage since they mainly rely on glycolysis.

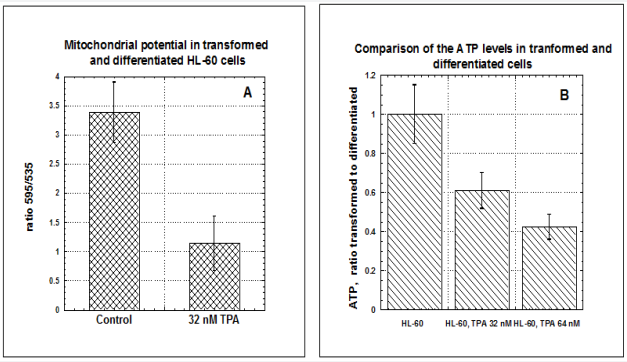

Figure 4: Effect of TPA on (A) mitochondrial potential (595:535 nm) and (B) cellular ATP (relative to control) in HL 60 cells.

The HL-60 leukemia model allows us to examine the effects of differentiation on mitochondrial metrics. This leukemia cell line normally grows in suspension in vitro. However, in the presence of 8 nM to 64 nM of 12-O-tetradecanoylphorbol 13-acetate (TPA), these cells will attach to plastic substrate, stop dividing, and undergo morphological changes. They also show increased expression of CD11a, consistent with differentiation to a monocyte or macrophage phenotype. Figure 4 shows the effects of TPA induced differentiation on mitochondrial potential (A) and ATP production (B). Undifferentiated HL-60 cells (Control) had significantly higher mitochondria potentials and cellular ATP levels. Again, part of this can be explained by changes in mitochondrial masses, which in transformed HL-60 cells were twice those in TPA differentiated HL-60 cells. We also examined the effects of oligomycin and CCCP on these cells, but the inhibitors were not significant compared to the effects of TPA.

Proliferating cells show higher levels of ATP production and higher mitochondrial potentials relative to non-proliferating cells. This is illustrated in Figure 5, where lung fibroblast cells (CCD-153) grown to confluence (total culture flask surface area coverage) in culture are compared to those given room to proliferate (twenty percent of flask surface area covered). The proliferating cells had mitochondrial potentials twice as high as those for confluent cells and ATP levels in proliferating cells were 2.5 times higher than those in confluent cells. A similar two-fold increase with proliferation was observed for mitochondrial mass, as measured by cardiolipin.

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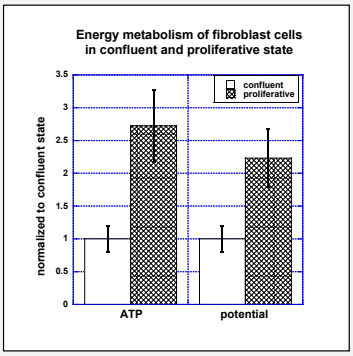

Figure 5: Mitochondrial potential and ATP levels for CCD-153 fibroblast cells: comparison of proliferating to confluent cells.

To summarize; tumor cells tended to have higher ATP levels per cell, more mitochondrial mass per cell and higher mitochondrial potentials relative to normal cells. Increased numbers of mitochondria is likely a primary factor in the increase of the other two parameters studied. ATP levels and mitochondrial potentials are also increased in proliferating cells, relative to confluent monolayers and in undifferentiated cancer cells, relative to differentiated cells of the same type. The increased mitochondrial mass and activity in cancer cells is also manifest in their ability to more closely maintain normal ATP levels in the face of the ATP synthase inhibitor oligomycin and the ionophore CCCP.A correlation analysis between ATP production and cardiolipin between normal and malignant cells, reveals that there is a high correlation (R=0.8) between ATP in and cardiolipin in the normal cells, while this correlation in malignant cells is relatively poor (R=0.33)(Figure 1a).

Discussion

For about a century it has been known that one of the most common properties of cancer cells is their ability to utilize and catabolize glucose at high rate. This metabolic alteration of cancer cells called the ‘‘Warburg phenomenon”, that is an increased flow through glycolysis in spite of high oxygen presence, which leads to enhanced lactate generation [37]. Even under conditions of plentiful oxygen, cancer cells still choose to switch to glucose fermentation with subsequent lactic acid formation. It has been explained by various possible mechanisms such as the metabolic adaptation to the hypoxic environment; by a direct effect of hypoxia-inducible factor on mitochondrial bioenergetics, mutations in oncogenes and proteins related to signal transduction pathways that interfere with mitochondrial bioenergetics and by mutations in mitochondrial DNA or in nuclear genes involved in the metabolic and bioenergetic functions. Nevertheless we favor the metabolic origin of this bioenergetic derangement as explained previously [19].

Today, the Warburg effect is regarded as the phenomenon of increased glycolysis in cancer cells even in the presence of oxygen, without a corresponding increase in oxidative phosphorylation. However, the original hypothesis claiming impaired mitochondrial function to be the cause of the glycolytic phenotype and the formation of cancer has been questioned. We again would like to resurrect the concept of cancer is a metabolic disease as also explained by Seyfried [22].

Our data are in agreement with other results that show that transformed and proliferative cells achieve high rates of glycolysis [38-40]. Our data also supports an increased glycolysis in transformed cells in comparison with normal cells [41]. In experiments with inhibition of ATP production by OxPhos by addition of CCCP, a pure uncoupler that acts as ionophore, completely dissipating the chemiosmotic gradient, but leaving the electrotransport system uninhibited, resulted in a decrease mitochondrial potential. The addition of CCCP with the antibiotic oligomycin or oligomycin alone, that acts by binding ATP synthase in such a way that blocks the proton channel, the ETC runs but no ATP synthesis occurs, the inhibition of ATP production was 60%-70% in normal cells in comparison 30%-40% in cancer cells showing that energy production in cancer cells is mainly from glycolysis.

The same results were found for transformed and differentiated leukemia cells HL-60. Cells were differentiated by incubation with different concentrations of TPA. As the result of differentiation the mitochondrial potential and ATP production were decreased 2-3 times. The evaluation of the contribution of glycolysis and OxPhos to the maintenance of ATP content showed the decreased level of ATP (approximately 2 times for the highest concentrations of inhibitors) in differentiated cells in comparison with the transformed cells.

To find if the shift to a glycolytic pathway is increased in cancer cells because glycolysis is required for cellular proliferation, we compared the levels of ATP and mitochondrial potential for normal fibroblast cells (CCD153) at the stages of confluence and proliferation. According to our data there was increase in the mitochondrial potential, mass and energy production in proliferative cells in comparison to confluent cells. It is interesting that the ratio of ATP and mitochondrial potential in confluent cells as compared to proliferative cells was the approximately the same as for cancer and normal cells. We also showed that the growth of tumorigenic and non-tumorigenic cells in typical cell culture media increase cardiolipin, the signature phospholipid of the inner mitochondrial membrane. According to our data the amount of these proteins correlated with mitochondrial potential (R=0.5).

Comparison of the levels of total mitochondrial potential (accumulation of JC-1 dye) and mass in transformed and normal cells showed the increased levels of these parameters in transformed cells that can be, probably, explained by proliferation of the cells. However, our data showed that the ratio of mitochondrial potential to mass was not statistically significant in malignant cells (leukemia cells and transformed fibroblasts) different from normal cells (T cells and fibroblasts CCD-153).

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We suggest that increased cardiolipin and the increased level of accumulation of JC-1aggregates in cancer cells can be explained by the increased number of aberrant mitochondria in proliferative cells [42]. Also in Figure 1(b) in which ATP production rates highly correlated with mitochondrial potentials with higher measures of ATP in normal cells in comparison with cancer cells at the same value of mitochondrial potential supports the same principle.

Several studies rule out the possibility that aerobic glycolysis is unique to cancer cells or that the Warburg effect only develops when oxidative capacity is damaged [43]. Indeed, many highly proliferative cancer cell lines that have been carefully studied do not seem to have defects in oxidative metabolism [43-45]. An explanation to these findings is that these studies have not considered mitochondrial substrate level phosphorylation that could give an impression that respiration is active when is not. It could be considered a form of "pseudo-respiration". This "pseudo-respiration" can also be achieved by Tri-carboxylic acid (TCA) cycle metabolism of Glutamine, which occurs in the mitochondria and not in the cytoplasm giving the false impression of an active oxidative metabolism [17].

According to the review [41], to synthesize lipids, proteins, and nucleic acids, cells use precursors derived from TCA cycle intermediates and a key role of the TCA cycle in proliferating cells is to act as a hub for biosynthesis. Nevertheless these can be provided by glutamine metabolism (substrate level phosphorylation) and not necessarily from oxidative respiration. This is an important difference related to the metabolism of transformed and normal cells. Authors of the review [41] examined the idea that several fluxes, including aerobic glycolysis, de novo lipid biosynthesis, and glutamine-dependent anaplerosis, support proliferation of diverse cell types. The regulation of these fluxes by cellular mediators of signal transduction and gene expression, includes the phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR system, hypoxia-inducible factor 1 (HIF-1), and Myc, during physiologic cell proliferation and tumorigenesis.

In particular, HIF-1 induces expression of pyruvate dehydrogenase kinase 1 (PDK1), which phosphorylates and inhibits the PDH complex [46,47]. This limits entry of glycolytic carbon into the TCA cycle and increases conversion of pyruvate to lactate. This adaptation may be important for cell survival during hypoxia. However, authors suggest that while HIF-1 stimulates glycolysis, and actively represses mitochondrial function and oxygen consumption, HIF-dependent mitochondrial changes are mainly functional.

The other suggestion is that the mechanisms that integrate signal transduction and cell metabolism are largely conserved between normal cells and cancer cells. The major difference is that in normal cells, initiation of signaling requires extracellular stimulation, while cancer cells often have damaged mitochondria and mutations that chronically enhance these pathways, allowing them to maintain a metabolic phenotype of biosynthesis independently of normal physiologic constraints.

According to several studies, mitochondria from tumor cells present evident ultra-structural alterations, are deficient in the b-F1 ATP synthase subunit, and are characterized by an increased $\Delta \Psi$ [48,49], altogether pointing to primary defects in the respiratory chain that may contribute to aerobic glycolysis. Mitochondrial potential required for mitochondrial ATP synthesis via F1FO-ATP synthase and many types of cancers exhibit increased mitochondrial potential, often coupled to inefficient OXPHOS [50,51].

Other studies have confirmed that restricting glycolysis or diverting pyruvate into the mitochondria, can significantly induce respiration in cancer cells [7,19,52]. These studies confirmed that the fate of pyruvate [either reduction in the cytosol by lactate dehydrogenase (LDH) or oxidation in the mitochondria by pyruvate dehydrogenase (PDH)] can determine the direction of tumor metabolism. The inhibition of lactate dehydrogenase or the activation of pyruvate dehydrogenase [via the inhibition of pyruvate dehydrogenase kinase (PDK)], can induce tumor cells to oxidize pyruvate in the TCA cycle and stimulate mitochondrial respiration [7,19,53]. It suggests that mitochondrial activity is not irrevocably impaired in cancer cells as thought by Warburg.

In the study [9] data shows that the defective mitochondrial system described in cancer cells can be dramatically improved by solely changing substrate availability and that HeLa cells can adapt their mitochondrial network structurally and functionally to derive energy by glutaminolysis only. This could also provide an explanation for the enhancement of oxidative phosphorylation capacity observed after tumor regression or removal. This work demonstrates that the pleomorphic, highly dynamic structure of the mitochondrion can be remodeled to accommodate a change in oxidative phosphorylation activity.

Conclusion

The cancer cell lines we examined tend to have higher mitochondrial potentials, cardiolipin levels, and ATP levels (on a per-cell basis) than the normal cell lines, with increased mitochondrial mass (as indicated by increased cardiolipin levels) being a major factor in elevating levels of the other two variables in cancer cells. This suggests that cancer cells seem to make up for the apparent insufficiency of aerobic respiration (in terms of ATP production) by increasing the glycolytic rate and, probably, by utilizing glutamine fermentation as an energy source.

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