Chapter 8

Is Mitochondrial Glutamine Fermentation a Missing Link in the Metabolic Theory of Cancer?

AMINO ACID FERMENTATION CAN MAINTAIN CELLULAR ENERGY HOMEOSTASIS DURING ANOXIA

Mitochondrial amino acid fermentation is known to maintain metabolic homeostasis under hypoxia in several species of diving animals (1, 2). Mitochondrial amino acid fermentation can also maintain metabolic homeostasis in the heart and kidney under low glucose and low O_2 conditions (1–5). The possibility that tumor cells might also obtain energy through amino acid fermentation has not been considered previously as an alternative energy source to OxPhos. Although Warburg considered respiration and glucose fermentation as the sole producers of energy within cells, amino acid fermentation in the mitochondria can also produce energy through substrate-level phosphorylation (1).

Schwimmer et al. (6) showed that the energy derived from TCA cycle substrate phosphorylation (succinyl-CoA synthetase step) (Fig. 4.6) was sufficient to compensate for F1-ATPase deficiency in yeast cells. It is unclear if Warburg was aware of energy that could be derived through this step, as he did not to my knowledge discuss this in his writings (7–11). Indeed, we were the first group to report that Krebs cycle substrate-level phosphorylation might compensate for insufficient respiration in metastatic cancer cells (12). On the basis of preliminary studies, I suggest that energy through glutamine fermentation could compensate for

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insufficient or suppressed respiration in those tumor cells that can use glutamine for energy.

While it is well known that glucose can be fermented, less is known about amino acid fermentation. Lactate is the by-product of glucose fermentation, whereas succinate, alanine, and aspartate are by-products of glutamine or amino acid fermentation under hypoxia (1–5). The expression of lactate in the presence of O_2 is abnormal and would indicate that the cells are fermenting. The degree of fermentation (lactate production) is positively correlated with the degree of malignant growth (10). Also, the less is the respiration, the greater is the fermentation. Under anoxia, fumarate can replace O_2 as an electron acceptor. If the cells consume oxygen, it is unlikely that succinate would accumulate. Under high glucose, amino acid fermentation can occur whether or not succinate accumulates. Hence, it is important to account for the multiple variables required to assure that cells are actually using OxPhos alone or are using some combination of OxPhos and mitochondrial substrate-level phosphorylation to maintain their viability.

EVIDENCE SUGGESTING THAT METASTATIC MOUSE CELLS DERIVE ENERGY FROM GLUTAMINE FERMENTATION

My graduate student, Roberto Flores, and I propose that glutamine and its metabolites (glutamate and α-ketoglutarate) could be fermented for energy in cancer cells under certain metabolic conditions, for example, under hypoxic or in high glucose under normoxia. High glucose levels suppress respiration through a Crabtree effect, thus producing increased fermentation. We presented evidence for this possibility at the 2011 meetings of the American Association of Cancer Research (13). We examined the influence of glucose and glutamine on ATP synthesis and viability in cultured mouse VM-M3 cells, a model for invasive human glioblastoma and systemic metastasis. These cells are known to have abnormalities in the content and composition of cardiolipin, which is linked to abnormal respiration (14).

Using a bioluminescent-based in vitro ATP assay, we found that ATP production and cell viability were similar in the metastatic cells grown in media containing either glutamine alone or glucose alone (Fig. 8.1). Shelton (15) also showed that lactate production was significantly lower in the metastatic cells grown in glutamine than in the cells grown in glucose, indicating that these cells produce little lactate from glutamine alone (Fig. 4.10). Recent findings from my graduate student, Linh Ta, showed that only trace levels of ^{14}C-labeled glutamine carbons are found in lactate when the VM-M3 cells are grown in 25 mM glucose (unlabeled) and 4 mM glutamine (radiolabeled). Significant radiolabeled lactate was found, however, when ^{14}C-labeled glucose was added. These findings indicate that very few glutamine carbons are present in lactate when high glucose is also present in the media.

However, ATP synthesis and lactate production were significantly greater in the VM-M3 tumor cells grown in glucose and glutamine than that for the tumor

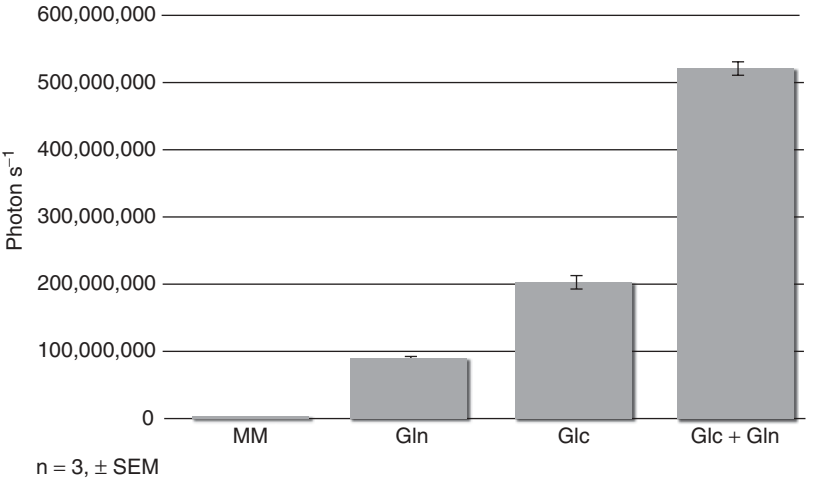

Figure 8.1 Effect of glucose and glutamine on viability of metastatic VM-M3 glioblastoma cells. In total, 5×10^4 cells were seeded in 96-well plates in 100 µl of DMEM plus 5% FBS and allowed to settle for 6 h before washing with 1 × phosphate buffered saline (PBS) with subsequent addition of minimal DMEM media containing either 25 mM glucose alone, 4 mM glutamine alone, or both metabolites together. After the cells were incubated for 24 h in 95% air and 5% CO_2, the Promega CellTiter Glo ATP assay was performed. Values represent the mean ± SEM of three independent samples per group. The results show that glucose and glutamine work synergistically compared to each metabolite alone (MM = minimal media). These data were presented at the 2011 meeting of the American Association of Cancer Research (13).

cells grown in either glutamine alone or glucose alone (Figs. 4.10 and 8.1). These findings show that glucose and glutamine work synergistically to enhance ATP synthesis, lactate production, and growth.

The synergy we found in the VM-M3 tumor cells was due to glutamine, as neither aspartate nor alanine (alternative nitrogen sources) could replace glutamine for the effect (Fig. 8.2). Previous results from Reitzer et al. (16) showed that the nonfermentable sugars, galactose and fructose, could replace glucose as a driver of energy metabolism in HeLa cells. Synergy for ATP synthesis and growth in the metastatic mouse cells arises from a specific interaction between glucose and glutamine metabolism. However, many tumor cells such as A549, HepG2, HeLa, U-87, U-251, and MDA-MB-453 can grow with minimal glucose. Many of these lines have a low glycolytic capacity relative to highly glycolytic tumors such as VM-M3, MCF-7, D-54 MG, GL 261, and 143B. All of the highly glycolytic cell lines are unable to grow without glucose.

We also collaborated with Cheryl Strelko and Mary Roberts in the Boston College Chemistry Department to further examine mitochondrial function in the metastatic mouse cells. Strelko and Roberts identified succinate, aspartate, alanine, and citrate in the tumor cells grown in pan-labeled glutamine using [C^{13}] NMR analysis (Fig. 8.3). These data were presented to support mitochondrial energy production through Krebs cycle substrate-level phosphorylation (12) and indicate

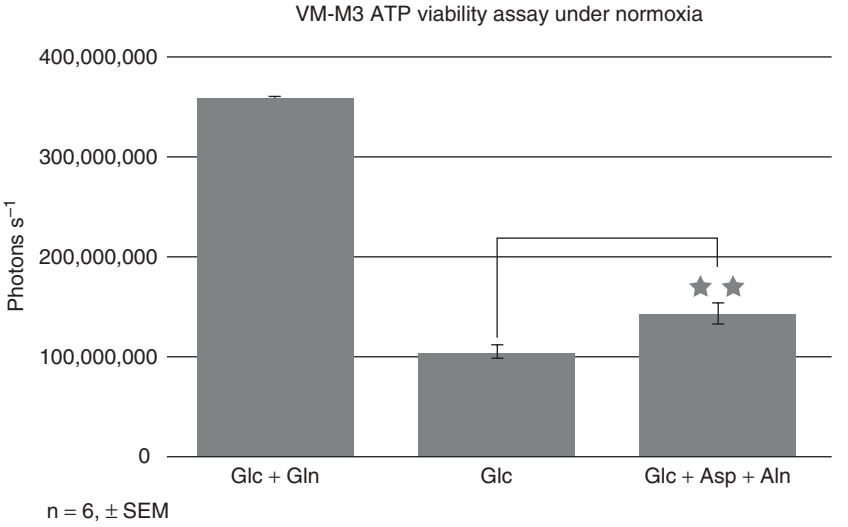

Figure 8.2 Glutamine is a better metabolic fuel for VM-M3 glioblastoma cells than are aspartate and alanine. Cells were grown in minimal DMEM media containing 25 mM glucose plus 4 mM glutamine, 25 mM glucose, or 25 mM glucose plus 4 mM aspartate and alanine. After the cells were incubated for 24 h in 95% air and 5% CO_2, the respective media were removed from each well and 100 µl DMEM plus 5% FBS were added followed by a 30-min equilibration to room temperature. ATP synthesis was measured as in Figure 8.1. Values represent the mean ± SEM of six independent samples per group. The asterisks indicate that the Glc+Asp+Aln values differ significantly from the Glc values at $p < 0.01$. The results show that neither aspartate nor alanine can substitute for glutamine as an energy metabolite in the VM-M3 cells. Other conditions are described in Figure 8.1. These data were presented at the 2011 meeting of the American Association of Cancer Research (13).

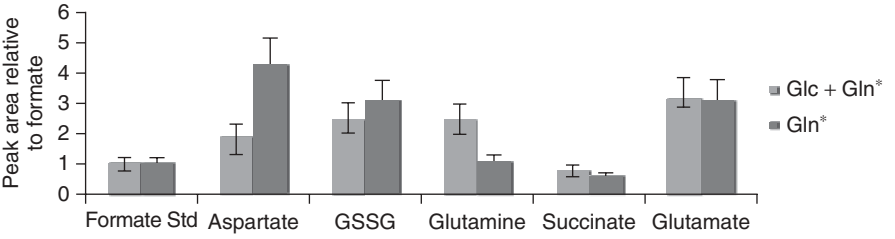

Figure 8.3 [C^{13}] NMR analysis of glutamine-labeled metabolites in VM-M3 glioblastoma cells. VM-M3 cells were grown in the presence of 4 mM C^{13} glutamine ± unlabeled 25 mM glucose. Cell extracts were collected via ethanol extraction after 12 h. Lyophilized extracts were redissolved in D_2O with 2 mM sodium formate standard and pH adjusted to 7.4. 1D-g HSQC (Heteronuclear Multiple Quantum Correlation) spectra were analyzed, and peaks were integrated with respect to the formate standard. Data are expressed as the average peak area relative to the formate standard of three independent samples ± the average % error. Asterisk indicates C^{13}. These data were presented at the 2010 meeting of the American Association of Cancer Research (12).

that glutamine is metabolized through the TCA cycle in these cells. In other words, mitochondria are capable of metabolizing glutamine in these tumor cells. The question arose as to whether these cells were using the glutamine to produce energy through OxPhos or through mitochondrial fermentation. The role for glutamine in energy production would be in addition to the known role of glutamine in replenishing TCA cycle metabolites (anapleurosis) (17, 18).

We showed that tumor cell viability and ATP production were robust in either anoxia or cyanide as long as both glucose and glutamine were present in the media (Figs 8.4 and 8.5). Since anoxia (95% N_2, 5% CO_2) or cyanide (an inhibitor of complex IV respiration) inhibits OxPhos, the robust synergy seen for glucose and glutamine is unlikely due to significant energy from OxPhos. Scott et al. (18) also found significant ATP production from glutamine under hypoxia in human melanoma cells but did not describe how ATP was formed from glutamine in the absence of O_2. We propose that the glucose/glutamine energy synergy observed in our metastatic mouse cells arises from linked fermentation redox couples in the cytoplasm and mitochondria that synthesize ATP largely through nonoxidative substrate-level phosphorylations.

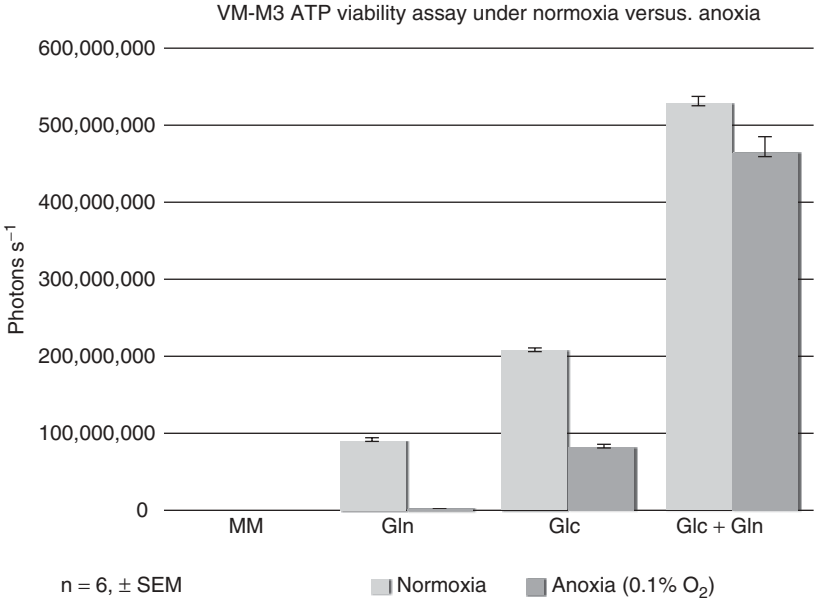

Figure 8.4 Influence of anoxia on viability of VM-M3 glioblastoma cells. Cells were grown in minimal DMEM media containing 25 mM glucose alone, 4 mM glutamine alone, or both metabolites together. After 24-h incubation of one 96-well plate in 95% air and 5% CO_2 and another in 95% nitrogen and 5% CO_2 (Biospherix Chamber), the ATP assay was performed. Values represent the mean SEM of three independent samples per group. Other conditions were as described in Figure 8.1. These data were presented at the 2011 meeting of the American Association of Cancer Research (13). To see this figure in color please go to ftp://ftp.wiley.com/public/sci_tech_med/cancer_metabolic_disease.

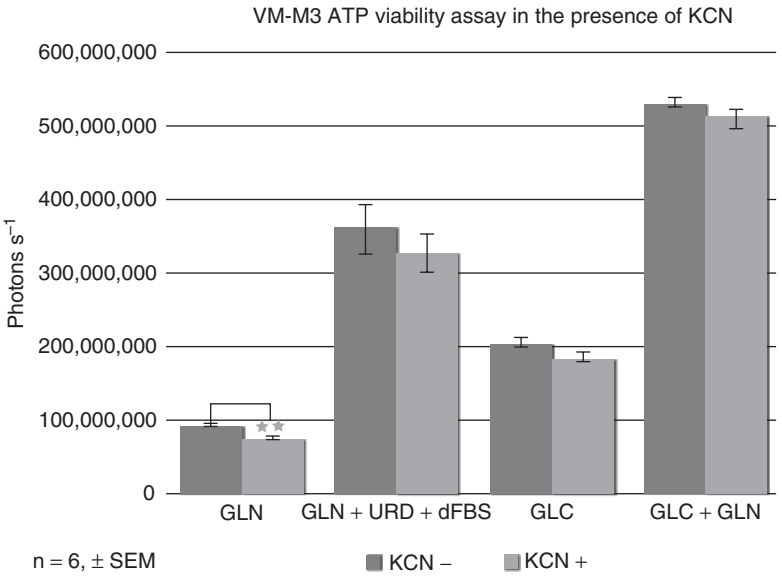

Figure 8.5 Potassium cyanide influence on viability of VM-M3. Cells were grown in minimal DMEM (Dulbecco's Modified Eagle Medium) containing 25 mM glucose alone, 4 mM glutamine alone, or both metabolites together as well as all these conditions plus 1 mM KCN. After 24 h of incubation in 95% air and 5% CO_2, ATP synthesis was measured as above. The asterisks indicate significant difference between Gln+KCN and Gln−KCN at $p < 0.01$. Values represent the mean ± SEM of three independent samples per group. URD = uridine and dFBS = dialyzed fetal bovine serum. The results from these data and those in Figure 8.5 show that OxPhos plays an insignificant role in VM-M3 energy metabolism when the cells are grown under anoxic conditions or in the presence of the complex IV inhibitor KCN. Other conditions were as described in Figure 8.1. These data were presented at the 2011 meeting of the American Association of Cancer Research (13). To see this figure in color please go to ftp://ftp.wiley.com/public/sci_tech_med/cancer_metabolic_disease.

FERMENTATION ENERGY PATHWAYS CAN DRIVE CANCER CELL VIABILITY UNDER HYPOXIA

Hochachka et al. (1, 2) presented compelling evidence for the existence of linked fermentation redox couples that could maintain energy homeostasis under hypoxia in metazoans and in diving animals. Several investigators showed that similar pathways could be used to maintain energy metabolism and cellular viability in heart and kidney under periodic hypoxia (3–5, 19–22). Tomitsuka et al. (23) recently provided the first evidence for the existence of this type of energy metabolism in cancer cells. Hence, cytoplasmic and mitochondrial amino acid fermentation could compensate for OxPhos under hypoxia. Many cancer cells can grow in hypoxic environments. Is it possible that cancer cells use energy through these pathways to compensate for respiratory injury? We suggest that they might, but this would occur only under specific conditions, for example, hypoxia or high glucose.

Our new concept to explain cancer cell energy metabolism is illustrated in Figure 8.6 and is a modification of the concept of Hochachka. We presented these pathways for the first time at the 2011 meetings of the American Association of Cancer Research (13). The malate–aspartate and glycerol 3-phosphate shuttles can link the redox couples in cytoplasm and mitochondria. This linkage is consistent with evidence showing high expression of these shuttle systems in various cancer cells (24–26). Shuttle expression in tumor cells, however, depends in part on whether cells can grow in the presence or absence of glucose.

In addition to the shuttles, the mitochondrial fumarate reductase pathway is also thought to produce ATP under certain hypoxic conditions (1, 23). NADH serves as the electron and proton donor, whereas fumarate serves as the ultimate electron and proton acceptor with succinate as an end product. Our model would be most relevant in those cancers that proliferate when using both glucose and glutamine to drive energy metabolism. The model would require modification to explain energy metabolism for those tumors that express defects in the TCA cycle and depend more heavily on glucose than glutamine for energy metabolism (28, 29).

According to our model, simultaneous glutamine and glucose fermentation would maintain cancer cell viability in those environments where oxygen is limited (hypoxia). It remains to be determined, however, if glutamine can also be fermented in tumor cells in the presence of oxygen. The Warburg effect involves the continued fermentation of glucose in oxygen. Aerobic lactate production provides this evidence. Succinate accumulation is indicative of amino acid fermentation under hypoxia. It is not yet clear if the succinate detected in the tumor cells under aerobic conditions from the NMR experiments results from glutamine fermentation. Succinate should not accumulate in cells that respire (1). It is also possible that glutamine is oxidized under aerobic conditions but is fermented under hypoxia.

Glutamine could also be metabolized under hypoxia through anaerobic respiration involving uncoupled electron transport. Elevated glucose levels would suppress OxPhos through a Crabtree effect, thus allowing the possibility of glutamine fermentation under normoxia. It would be difficult to distinguish glutamine respiration from glutamine fermentation under normoxia since both processes would involve electron transfer and TCA cycle activity.

Glutamine fermentation, occurring under high glucose conditions, will generate considerable energy through substrate-level phosphorylation and possibly through the fumarate reductase reaction (1, 2). Neither process involves OxPhos but would still require uncoupled electron transport. ATP uptake into the mitochondria from the cytoplasm and electron transport would be needed to drive the F1-F0-ATPase in reverse in order to maintain a proton motive gradient (2, 30). We think this situation would be present in those highly glycolytic tumor cells where the hexokinase-2 becomes attached to the outer mitochondrial membrane as described by Pedersen (31). The ATP needed to drive the ATP synthase in reverse under hypoxia would come almost exclusively from glucose and glutamine fermentation. *Hence, targeting glucose and glutamine could effectively shutdown energy metabolism in many cancers that depend on these metabolites for energy*.

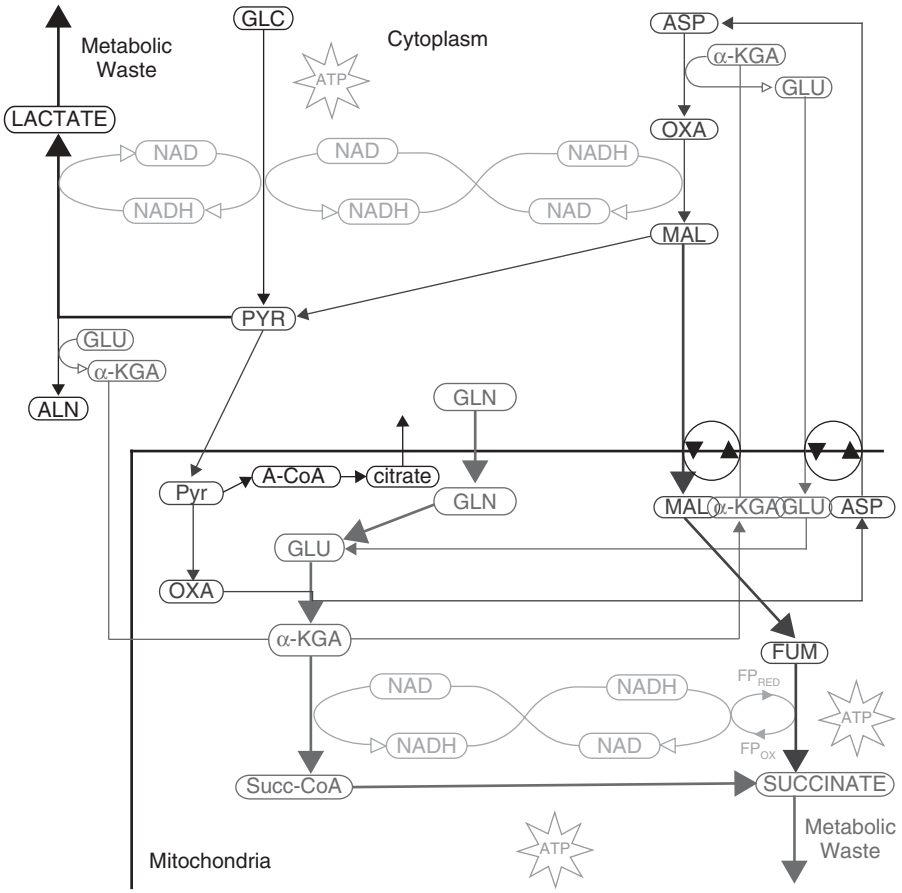

Figure 8.6 Proposed pathways for fermentation energy metabolism in VM-M3 glioblastoma cells. Fermentation redox couples formed in the mitochondria and cytoplasm can generate ATP under hypoxia. Since cancer cells are known to be in a state of pseudo-hypoxia, the proposed schematic is logical. In the mitochondrial fermentation scheme, the fumarate reductase (FRD) system presides, as fumarate rather than oxygen becomes the final electron acceptor. The activity of the aspartate–malate shuttle can link the cytoplasmic and mitochondrial redox couples under hypoxia. Glucose-derived pyruvate is considered the sole source of lactate and alanine. For each mole of alanine "bled off" the glycolytic pathway, 1 mole of NAD+ must be generated from a source other than lactate dehydrogenase. The redox imbalance in the glycolytic pathway can be corrected by reduction of aspartate-derived oxaloacetate to malate. The malate and α-ketoglutarate formed in the cytoplasm can then be transported into the mitochondria in exchange for other anions (27). We believe that this mechanism also provides cancer cells energy under normoxia when high glucose is also present in the media. Under normoxia and high glucose, oxygen would replace fumarate as the electron acceptor and the F1F0-ATPase would run in reverse. This would be linked to attachment of hexokinase-2 to the mitochondria (refer to text for more detail). This metabolic pathway was presented at the 2011 meeting of the American Association of Cancer Research (13). See color insert.

Warburg was aware of the difficulty in attempting to shutdown tumor energy metabolism in the body (11). Restricting availability of glucose and glutamine becomes a simple and effective therapeutic strategy for cancer management. I address in Chapter 17 how we can shutdown tumor energy metabolism in vivo using combinations of energy-restricted ketogenic diets and drugs that target glucose and glutamine metabolism.

Tumor cells survive in hypoxia "not" because they have a growth advantage over normal cells but because they can ferment organic molecules. Organic molecules become O_2 surrogates in accepting electrons. Cancer cells not only ferment glucose, as Warburg first showed, but they might also ferment glutamine and possibly other amino acids in the mitochondria under hypoxia and when glucose levels are high under normoxia. Unlike normal cells that can switch back to OxPhos when O_2 becomes available, most tumor cells depend on fermentation metabolism whether or not O_2 is present in the environment. Tumor cells adapt to fermentation because their OxPhos is insufficient to maintain energy homeostasis. Fermentation adaptation underlies the pathology of cancer.

The failure to consider amino acid fermentation as an alternative energy source for tumor cells can cause confusion regarding energy metabolism in cancer. It can be difficult to distinguish the effects of glutamine oxidation from glutamine fermentation since both processes occur in the mitochondria. The difference between glutamine oxidation and glutamine fermentation is that the latter does not couple the proton motive gradient to ATP production. Warburg was also unaware of this energy source in tumor cells, as he considered residual OxPhos activity as the likely origin of the low aerobic ATP production in cancer cells (7, 8). We also do not exclude this possibility, as it remains to be determined if glutamine is fermented or oxidized under normoxic when glucose levels are low. Residual glutamine oxidation coupled with detectable but low glycolysis could occur in low glycolytic tumor cells. As Warburg mentioned, however, no tumor cells are known that do not ferment at least some glucose indicative of respiratory insufficiency (10).

COMPETING EXPLANATIONS FOR THE METABOLIC ORIGIN OF CANCER

Currently, I consider that there are three major hypotheses regarding the role of energy metabolism in the origin cancer cells. The first hypothesis is that of Weinhouse, which considers that cancer cells express aerobic glycolysis despite having normal respiratory function. The evidence for this view was presented in Chapter 6. This view is also consistent with the gene theory of cancer in that abnormalities in oncogenes and tumor suppressor genes are ultimately responsible for aerobic glycolysis. More specifically, gene defects cause aerobic glycolysis and the metabolic defects seen in cancer cells. Dang and colleagues summarized this view in their recent paper where they stated: "*Today, we understand that the relative increase in glycolysis exhibited by cancer cells under aerobic conditions was mistakenly interpreted as evidence for damage to respiration instead of damage to the regulation*

of glycolysis" (32). According to this view, abnormal expression of oncogenes and tumor suppressor genes are ultimately responsible for glycolytic damage and the metabolic reprogramming of cancer cells.

This view of cancer origin is at odds with the metabolic theory that insufficient respiration is the origin of cancer. Warburg argued that damaged respiration is more common in cancer cells than is damaged fermentation. Respiration is more complicated than fermentation because it requires mitochondrial structure and many more enzymatic steps than glycolysis (9, 33). Warburg stated, "*it is one of the fundamental facts of present-day biochemistry that adenosine triphosphate can be synthesized in homogeneous solutions with crystallized fermentation enzymes, whereas no one has succeeded in synthesizing adenosine triphosphate in homogeneous solutions with dissolved respiratory enzymes, and the structure always goes with oxidative phosphorylation*" (8). Simply put, respiratory damage is more likely in cancer than is damage to fermentation (glycolysis).

In order to accept the Weinhouse hypothesis, one would need to overlook or discount the massive data of Pedersen and others (presented in Chapters 5–7) showing that mitochondrial structure and respiration are damaged in cancer cells. In addition, one would need to ignore or overlook the evidence from the nuclear/cytoplasmic transfer experiments showing that normal mitochondria can reprogram cancer nuclei to form normal tissues (covered in Chapter 11). However, normal nuclei are unable to reprogram the tumor cytoplasm to form normal cells. These experiments rule out a chromosomal (somatic mutation) origin of cancer and strongly implicate the importance of extrachromosomal, nonnuclear systems (mitochondria).

The second hypothesis suggests that elevated glycolysis suppresses respiration in cancer cells. Under this hypothesis, cancer respiration is considered repressed, but the repression arises secondary to the appearance of aerobic glycolysis. In other words, many of the abnormalities seen in tumor mitochondria structure and function would arise as effects rather than the cause of aerobic glycolysis. The findings of the Cuezva, Mazurek, and Rossignol groups seem to support variations of this hypothesis (34–37). While this hypothesis is consistent with many of Warburg's findings, this hypothesis also seems in line with the genetic origin of cancer, as abnormalities in oncogenes and tumor suppressor genes are thought responsible for elevated tumor glycolysis. To accept this hypothesis, one would also need to overlook the evidence from the nuclear/cytoplasmic transfer experiments showing that extrachromosomal processes, rather than nuclear mutations, drive tumorigenesis.

In contrast to the first two hypotheses, we favor Warburg's original hypothesis with the caveat that tumor cells can also use mitochondrial fermentation in addition to glycolysis to compensate for insufficient respiration. While the evidence supporting our hypothesis is still preliminary, I believe that this will help clarify the metabolic origin of cancer. It is my opinion that the view of cancer as a nuclear gene-driven process has stymied investigations into the mitochondrial origin of the disease. Our hypothesis can accommodate most characteristics of cancer, once the nuclear gene origin of the disease is rejected. Consequently, a critical reevaluation

of the gene theory of cancer is necessary before the metabolic theory of cancer can be fully appreciated. I cover this reevaluation in Chapters 9–11 and 15.

CHAPTER SUMMARY

Cancer is a disease of abnormal energy metabolism. In order to survive with insufficient respiration, tumor cells have adapted to energy production through fermentation. Powerful synergy is established between fermentation redox couples in the cytoplasm and mitochondria. These redox couples are linked through shuttle systems that drive tumor cell energy metabolism using glucose and glutamine as fermentable metabolic fuels. Adaptation to fermentation allows tumor cells to survive and grow in hypoxic environments. The information covered in this chapter raises the specter of mitochondrial glutamine fermentation as an energy source for tumor metabolism under certain conditions.

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