Warburg Effect or Reverse Warburg Effect? A Review of Cancer Metabolism

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\textbf{The Warburg Effect}

During his research on the energy metabolism of tumors, Otto Warburg found that cancer cells prefer to produce ATP (adenosine triphosphate) by glycolysis rather than by oxidative phosphorylation (OXPHOS) even in the presence of ample oxygen [1]. This finding was later termed the Warburg effect. The hypothesis is that cancer cells undergo increased glycolysis even in the presence of oxygen, a process also known as ‘aerobic glycolysis’. Warburg’s original study promptly caught the attention of the scientific community.

The scientific community then demonstrated that this hypothesis held true in different types of tumors, and successive test results further proved its unshakable status. Carl and Gerty Cori [2] confirmed that an increased glucose consumption was found in cancer cells with a shift from respiration to fermentation. A landmark conclusion was reached with the discovery that the anaerobic glycolysis rate in carcinoma cells was much higher than in normal tissues [3]. Advanced technologies such as [4] magnetic resonance spectroscopy and fluorodeoxyglucose positron emission tomography (FDG-PET), which were widely adopted in clinical research, greatly accelerated the study of energy metabolism and helped build the outline of the aerobic glycolysis system. Its main characteristics are an increased glucose uptake and synthesis of glycogen, lactate production and induced acidosis, which lead to an acid-mediated tumor invasion and the impairment of mitochondrial function in cancer cells [5].

\textbf{Keywords}

Glycolysis · Energy metabolism · Cell transformation, neoplastic

\textbf{Summary}

Cancer is a major threat to human health. A considerable amount of research has focused on elucidating the nature of cancer from its pathogenesis to treatment and prevention. Tumor cell metabolism has been considered a hallmark of cancer. Cancer cells differ from normal cells through unlimited cell division, and show a greater need for energy for their rapid growth and duplication. Research on glycometabolism, as the key point of energy metabolism, has played a unique role. In the 1920s, Warburg found that cancer cells prefer to produce adenosine triphosphate (ATP) by glycolysis, which is a less efficient pathway compared to oxidative phosphorylation. This striking discovery, called ‘the Warburg effect’, has influenced and guided the study of the mechanism and treatment of tumors for generations, but its causal relationship with cancer progression is still unclear. Some studies have now shown contradicting evidence and a new hypothesis, the reverse Warburg effect, has been put forward, in which cancer cells produce most of their ATP via glycolysis, even under aerobic conditions. In this review we discuss the new points concerning the energy metabolism of a tumor, as well as the current facts and perspectives.
Characteristics of the Warburg Effect

Increased Glucose Uptake and the Synthesis of Glycogen in Cancer Cells

Warburg described an increased glucose uptake in cancer cells, which this was considered the basis of cancer cell metabolism [6], and was subsequently the most used characteristic to distinguish tumor cells from normal cells. Cancer cells take up extracellular glucose, mainly via glucose transporters (GLUTs) of which there are 6 different subtypes. Different tissues share unique capacities to absorb and utilize glucose in cell metabolic pathways, especially in glycolysis. Once inside the cell, the glucose is converted to glycogen. An increased glucose uptake can, therefore, drive a strong synthesis of glycogen, and studies have demonstrated a notable content of glycogen in tumors.

Glycogen synthase, which is involved in glycogen synthesis, can be inactivated through the phosphorylation pathway. A multifunctional regulatory enzyme, glycogen synthase kinase-3 (GSK3), is crucial during this process. Its activity is regulated by insulin. GSK3 can be inhibited by the PI3K/Akt signaling pathway, which can be activated by related growth factors and insulin. GSK3 thus plays an important role as a tumor suppressor, affecting the metastatic potential and apoptosis of the cancer cells.

The pentose phosphate pathway (PPP) also plays an important role in glucose metabolism, in parallel to glycolysis. Nicotinamide adenine dinucleotide phosphate (NADPH) and ribose 5-phosphate are produced from a series of reactions, starting from glucose 6-phosphate. NADPH and ribose 5-phosphate are very important intermediate products of glycolysis. Studies using MCF10 cells (a normal human mammary epithelial cell line) suggested that the glucose flux through the PPP pathway is increased in cancer cells [7].

Lactate Production and Subsequent Acidosis

Increased lactate production causes a lowering of the pH. In 2002, Stern and Shuster [8] showed that the production of hyaluronan and the expression of CD44 can be stimulated by lactate. CD44 is the major transmembrane receptor for hyaluronan, and hyaluronan and the expression of CD44 can be stimulated by lactate. CD44 thus plays an important role as a tumor suppressor, affecting the metastatic potential and apoptosis of the cancer cells.

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Fischer and Hoffmann [9] reported that, to maintain the intracellular pH and simultaneously to produce cytokines, cytotoxic T lymphocytes co-transport H+ and lactate. The increased extracellular lactate resulting from the accumulation of cytotoxic T lymphocytes in the vicinity of cancer nidus disturbs the function and metabolism of T cells, allowing immune escape may occur. Ercinska [10] found that the NADH pool is depleted and the pyruvate dehydrogenase (PDH) inhibited during the progress of the lactate production as a result of the local lowering of pH. Lactate production may well be a positive feedback control mechanism in cancer metabolism. The intracellular pH would be decreased due to the overproduction of lactate, and then PFK-1 (6-phosphofructo-1-kinase), a rate-limiting enzyme in glycolysis, would be inhibited. The end result is glycolysis.

Damage to Mitochondrial Function

Cellular OXPHOS is a major metabolic pathway in mitochondria but can also produce reactive oxygen species (ROS), free radicals that can damage cells. Overproduction of ROS can initiate damage to macromolecules and to intracellular and extracellular signaling pathways, which can lead to cancer development. Lopez-Lázaro [11] hypothesized that cancer cells have a unique metabolism. Normal cells prefer to produce water and carbon dioxide via OXPHOS using oxygen and glucose, but oxygen is also converted to ROS, such as H2O2 (hydrogen peroxide) and O2− (superoxide), inducing glycolysis in cancer cells, and ROS formation can damage mitochondrial function. Furthermore, H2O2 can enhance the activity of Na+/H+-ATPase, and heighten the expression of proto-oncogenes. O2− formation leads to alkalinization in the cancer cells, depleting protons and stimulating PFK-1.

A mitochondrial uncoupling protein UCP2 has been shown to be overexpressed in a few drug-resistant cancer cell lines [12], and many scientists consider that the enhanced glycolytic ratio in cancer cells is due to this mitochondrial uncoupling protein. It has been suggested that apoptosis in cancer cells induced by various antitumor drugs was significantly lower because of chemoresistance as a result of the overexpression of UCP2 [13].

Challenges of the Warburg Effect

Warburg’s own experimental results were challenged during the year of their publication. His findings were critically analyzed by Weinhouse [14], who indicated that the respiratory rates of cancer cells were indeed as high as those of normal cells. This threw down the gauntlet to the Warburg effect as the example of cancer cell metabolism.

Recently, with the advent of high-resolution respirometry techniques and specific sensitive fluorescent probes, an overwhelming amount of data on respiratory rates and mitochondrial membrane potential from numerous cancer cell lines has become available. These reveal the active function of mitochondria in cancer cells. Furthermore, selective and sensitive tumor detection and imaging using copper-based lipophilic organic cations [14] or [18F]fluoro-glutamine [15] as PET probes that target mitochondrial metabolism have now been developed. These analytical techniques, along with gas/liquid chromatography-mass spectrometry have greatly enhanced the precision with which metabolic flows can be accurately assessed to determine the sources and exchange processes of various chemical subgroups as they are used in the different enzymatic reactions.

Nowadays it is argued that cancer cells do not have a greater advantage in glycolysis than normal cells [16]. Research on energy metabolism in various kinds of cancer cells compared to normal cells has suggested that, in general, normal cells do not rely less upon zymolysis than cancer cells. Nevertheless, some cell types, for instance, enucleated cells, erythrocytes and reticulocytes, or platelets, which are the fragments of megakaryocytes, were considered normal, but have unique energy metabolism [17].
Functions of Cancer-Associated Fibroblasts

In 2011, Martinez-Outschoorn and his team from the Jefferson Stem Cell Biology and Regenerative Medicine Center in UK used both co-culture and single cell culture methods with the fibroblasts and cancer cells, and compared the corresponding parameters of metabolism [18]. In a 5-day cultures, they found that with single cell cultures, fibroblasts and cancer cells shared parallel glucose uptake, and that cancer cells showed twice the mitochondrial activity. They also found that the ROS production in both kinds of cells was similar. Furthermore, under the conditions of co-culture, glucose uptake in fibroblasts was quadrupled compared to that in cancer cells, and the mitochondrial activity of fibroblasts was half that of cancer cells. In addition, ROS production in fibroblasts was tripled compared to that in cancer cells. They concluded that, under the conditions of co-culture, compared with fibroblasts, cancer cells shared a significant increase in mitochondrial activity, and that glucose uptake and ROS production were decreased in compensation. In other words, fibroblasts showed a decreased mitochondrial activity, increased glucose uptake, and increased ROS production. These conclusions suggest that cancer-associated fibroblasts may have aerobic glycolysis.

Structural Integrity and Unmarred Function of Mitochondria

When cancer cells are cultured under normoxia (i.e. 21% atmospheric O$_2$ equivalent to 50–100 μM dissolved O$_2$) and hyperglycemia (25 mM glucose), representing the most commonly used culture conditions, the contribution of glycolysis to the ATP supply are low, ranging from 9% to 25%. As mentioned above, higher contributions of glycolysis to the ATP supply in cancer cells have been described. When the cells are grown under severe hypoxia (i.e. 0.1% atmospheric O$_2$, which is equivalent to 10–15 μM dissolved O$_2$) and hyperglycemia, glycolysis predominates over OXPHOS for supplying ATP.

Although glycolysis predominates as the main ATP supply in mature MCTSs (multi-cellular tumor spheroids), anti-glycolytic treatment only has a marginal effect on inhibiting tumor proliferation, suggesting that cells inside the spheroids remain significantly dependent on the alternative ATP supply derived from mitochondria. This last proposal was assessed by disaggregating breast MCF-7/MCTSs, in which the 2 cell layers constituting whole spheroids (the external and proliferative layers and the inner and quiescent layers) were carefully separated before the energy metabolism of each fraction was evaluated and compared. Glycolytic rates in both MCTS cell layers were similar, but 2–3 times higher than those determined for the MCF-7 cells grown as a 2-dimensional monolayer. In contrast, OXPHOS rates in both MCTS layers, determined under high O$_2$, were 5–18 times higher than the corresponding glycolytic rates. Consequently, it follows that a higher ATP supply was emanating from the mitochondria, and would help to explain the greater susceptibility of MCTSs to mitochondrially targeted anti-cancer drugs [19].

Some researchers have implied that Gln transformation into 2-OG (oxoglutarate or ketoglutarate) does not require active respiration and OXPHOS because 2-OG derived from Gln only serves for anabolic purposes [20–22]. Thus, it has been concluded that the Warburg phenotype prevails, i.e. when Gln is being actively consumed, glycolysis is elevated and the Krebs cycle is altered in cancer cells to overproduce and extrude citrate from mitochondria. However, it is necessary to understand that to feed the anabolic pathways required for cancer cell growth, the 2-OG derived from Gln has to be transformed into malate and later into citrate through the forward Krebs cycle, or directly to citrate via the reverse reaction of isocitrate dehydrogenase (ICD). In the former case, a point that is often overlooked is that NADH is formed by 2-OGDH (2-oxoglutarate dehydrogenase) and MDH (malate dehydrogenase), which requires re-oxidation to NAD$^+$ by the respiratory chain for the continuous supply of malate and citrate. In other words, the active consumption of Gln by cancer cells requires a functional Krebs cycle and respiratory chain, regardless of whether this amino acid is used for catabolic or anabolic purposes. It has also been proposed that mutations in some of the Krebs cycle and respiratory chain enzymes disable OXPHOS [23]. However, this assumption requires experimental demonstration by directly determining OXPHOS flux, because many mutations may have no effect on enzyme activity and pathway function, i.e. a mutation may not significantly affect the kinetic properties of the enzyme/transporter, and altering an enzyme (e.g. by decreasing its $V_{max}$) may not have effect on the pathway flux if its control coefficient on the flux is negligible. This means that many point mutations can be silent because they have no effect on the kinetic properties of the pathway-controlling steps.

Experimental evidence indicates that the Km value for O$_2$ for respiratory complex IV (cytochrome c oxidase) in normal cells and mitochondria is in the range of 0.1–0.8 μM [24], whereas the range of O$_2$ concentrations reached under hypoxia in the center of solid tumors and MCTSs is 2.5–10 mmHg equivalent to 3–13 μM dissolved O$_2$ [25, 26]. Thus, there is no oxygen limitation of the respiratory chain activity in the hypoxic tumor regions. Consequently, other transcriptional and post-translational mechanisms or mitophagy must operate to promote the observed OXPHOS depression induced by chronic and severe hypoxia in cancer cells [27–30].

Metabolic Control Analysis of the ATP Supply in Cancer Cells

Analysis of the control of the ATP supply in cancer cells would be helpful in understanding the metabolism of cancer cells, in which 3 main processes involved: 2 pathways synthesizing ATP (glycolysis and OXPHOS) and 1 process consuming ATP (cellular work).

Monolayer cancer cells and MCTSs exhibit high proliferation rates when exposed to high O$_2$ and glucose, whereas growth is arrested after 24–48 h of hypoxia [28, 31, 32]. If OXPHOS is the predominant ATP supplier in cancer cells that grow actively, it is suggested that the control of the ATP supply must be shared by OXPHOS and the ATP demand. On the other hand, if glycolysis supplies most of the ATP in quiescent cells, then it is likely that glycolysis maintains control of the ATP supply, because the other 2 processes/components of the system are attenuated. These 2
proposals have relevant therapeutic implications. It is clear that drugs exclusively targeting either glycolysis or OXPHOS will not be able to achieve the goal of killing all cells within a solid tumor. Therefore, targeting both energy pathways seems mandatory. This can be attained by using multi-site drugs such as the copper-based anti-cancer drugs termed casiopeinas, which inhibit glycolysis at the hexokinase level [33] and OXPHOS at the PDH2-OGDH and SDH levels [34, 35]. However, the use of less-specific drugs may bring about undesirable side effects such as cardio-, nephro- and neurotoxicity, and anti-angiogenic activity affecting endothelial cells. Therefore, a more adequate strategy could be to target the principal controlling steps in each pathway/biological function, which additionally may differ between normal and tumor cells [36–39]. Furthermore, elasticity analysis of OXPHOS in intact rat hepatoma AS-30D cells showed that most of the control of flux resided directly within the pathway (66%), although significant control existed outside the pathway (34%), and was related to the ATP demand. Complementary studies of OXPHOS flux by titrating with specific and permeable inhibitors in the same cells established that the respiratory chain complex I (C I) exerted significant flux control (30%), which resulted from a comparably lower C 1 protein content present in these cancer cells versus normal hepatocytes [40].

Additional examples of the inadequacy of such assessments were seen when non-physiological conditions are used. When HepG2 cells (liver hepatocellular cells) were incubated with oligomycin (to fully inhibit OXPHOS) or valinomycin (to collapse the electrical membrane potential), the flux control exerted by C IV was decreased [41]. Although these last experimental conditions were used to unveil enzyme regulatory mechanisms, the flux-control values obtained probably have little or no physiological value because in situ the mitochondria (i.e. mitochondria inside living cells) maintain high H⁺ gradients and OXPHOS rates, which greatly affect the outcome for such studies. In studies using digitonin-permeabilized HepG2 cells, the flux control by C IV on ADP-stimulated respiration was 0.3 with Pyr plus Mal as oxidizable substrates, and 0.66 with Pyr + Mal + Succ [42]. The Ki value for cyanide used in the calculation of these flux-control values was 25 μM, which was determined in cells treated with an H⁺ ionophore to ensure that the mitochondria were unable to maintain their H⁺ gradient and thus achieve maximal rates of respiration. This Ki value is 2–10 times higher than those determined in coupled mitochondria, and hence it is possible that analysis of flux control determined using cyanide titration of C IV may in fact have much higher values if a lower Ki is used, reflecting the more physiological situation.

In human breast cancer biopsies permeabilized with saponin, the rate of ADP-dependent O₂ consumption (i.e. OXPHOS) was strongly controlled by the 6 evaluated steps: the respiratory chain complexes with flux-control coefficients of 0.44–0.46 for C I; 0.51–0.54 for C III; 0.73 for C IV; of 0.65 for ATP synthase; 1–1.03 for the adenine nucleotide translocator (ANT); and 0.58–0.65 for the Pi carrier [43]. As the sum of the flux-control coefficients was near 4 instead of 1, it was suggested that the natural molecular arrangement of the OXPHOS enzymes in supra-molecular complexes is preserved when intact cells or cells permeabilized with mild detergents are used, whereas the integrity of the supra-molecular complexes is not maintained when isolated mitochondria are studied.

**Conclusions**

There is much evidence that the Warburg effect has many questionable points. Based on a mass of research, a new hypothesis is catching people’s attention, the reverse Warburg effect. Glycolysis occurs in mesenchymal stoma cells under the activation of neighboring cancer cells. Furthermore, an increased formation of recycled nutrients is produced. This high-energy metabolism is transferred to the neighboring cancer cells by the orientation of transport to participate in the TCA cycle. The consequence is that the OXPHOS increases enhancing ATP production, thus constituting metabolic coupling (Fig.1). This new model may well explain both the way ATP is produced via a low efficiency method despite extremely high energy demand of the tumor cells, and reasonably explain the ‘autophagy paradox’ that has long been questioned. Although our focal point on the aerobic glycolysis mirrors the core of the realm, further research is still required on cancer bioenergetics.

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**Disclosure Statement**

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