Mitochondria and Cancer: The Warburg Fact

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Abstract

Mitochondria play a key role in the energy generation of cells. Here, we reassess the opportunities to fight cancer by manipulating the expression of mitochondrial DNA (mtDNA). The mtDNA encodes 13 polypeptides that are all critical for oxidative phosphorylation. Most cancers, if not all, use glycolysis as main bioenergetic pathway, despite the presence of oxygen. This is known as the Warburg effect and leads to disturbance of the mitocoplasmic energy balance. Cytosolic ATP levels are kept high by the increased glycolysis, limiting the demand for ATP from mitochondria. The restricted ADP-ATP exchange across the mitochondrial membranes results in a high ATP/ADP ratio within the organelles and a high mitochondrial membrane potential. Together, these increase the cancer cell’s resistance to apoptosis. Although the increased glycolysis may enhance the survival of cancer cells, several lines of evidence suggest that mitochondrial activity remains indispensable for proliferation. Specific inhibition of mitochondrial protein synthesis, e.g. with doxycycline, results in a mitonuclear protein imbalance, decreasing the apoptotic threshold and preventing proliferation of various cancer types in vivo. The anti-cancer effects are achieved at serum levels that are present in patients treated with the antibiotic to combat infections. There is good evidence to consider further clinical investigations with doxycycline to substantiate its beneficial effects on cancer.

Keywords: Actinonin; Cancer; Doxycycline; Mitochondrial Protein Synthesis; Tetracycline; Warburg effect

Abbreviations

DCA: Dichloroacetate; ΔΨm: Mitochondrial Transmembrane Potential; MtDNA: Mitochondrial DNA; oxphos: Oxidative Phosphorylation; PDC: Pyruvate Dehydrogenase Complex; PDK: Pyruvate Dehydrogenase Kinase; PPP: Pentose Phosphate Pathway.

Introduction

Mitochondria, antibiotics and cancer

In mammalian cells, the mitochondrial genome (mtDNA) is a circular molecule encoding indispensable genetic information. Every cell contains a substantial number of mtDNA copies that may be identical (homoplasmic) or heterogeneous (heteroplasmic). Mitochondria are evolutionary derived from ancient prokaryotic organisms of the α-proteobacterial type. Only a small number of genes has been preserved on mtDNA: 24 genes coding for the two rRNA and 22 tRNA species engaged in mitochondrial protein synthesis, and 13 genes coding for subunits of the mitochondrial ATP generating enzyme complexes of the oxidative phosphorylation (oxphos) system. The nucleus is the genetic basis for all other mitochondrial components.

Mitochondrial protein synthesis has retained some typical bacterial properties. For instance, akin to bacterial protein synthesis, mitochondrial protein synthesis starts with N-formylmethionine [3-5]. Nascent polypeptides may then be deformylated by a mitochondrial deformylase and, subsequently, demethionylated by a mitochondrial methionylaminopeptidase. The naturally occurring antibiotic actinonin is not only a potent inhibitor of bacterial peptide deformylases, but also of mitochondrial peptide deformylases [6]. Similarly, mitochondrial ribosomes are sensitive to antibacterial agents interfering with ribosomal functions, such as tetracyclines [7].

In the 1980s, we found that tetracyclines cause a proliferation arrest of tumor cells in vitro as well as in vivo [8,9]. In our experiments, proliferation arrest was always preceded by a marked decrease of the mitochondrial energy generating capacity [10]. We evaluated the mito-nuclear protein imbalance as result of treatment with tetracyclines by measuring the ratio of cytochromes associated with mitochondrial (cytochromes aa3) or nuclear-encoded (cytochromes c +c1) components of the oxphos complexes. Treatment of rats with continuous intravenous infusion of oxytetracycline for periods of up to 6 weeks, reaching serum levels of ~10 μg/ml, significantly decreased the aa3/c+c1 ratio in liver mitochondria [11]. Similar results were obtained at 5 μg/ml of the tetracycline analog doxycycline for tumors in athymic (nude) rats transplanted with NC-65, a tumor line derived from a human hypernephroma [8]. More recently, Lee and colleagues [6] showed that actinonin inhibited tumor cell growth in vitro and in vivo. Actinonin treatment led to tumor-specific mitochondrial membrane depolarization and ATP depletion in a time- and dose-specific manner. Collectively, these results suggest that disruption of the cell’s ability to produce mature mtDNA-encoded proteins has significant antitumor effects in a range of tumor systems.

Mitochondria and apoptosis

Mitochondria play a key role in apoptosis and necrosis [12]. Permeability transition of the pore complex of the inner mitochondrial membrane is regarded the coordinating event. A drop in mitochondrial transmembrane potential, ΔΨm, starts a cascade of reactions leading to apoptosis. Through interruption of the synthesis of mature mitochondrial proteins, actinonin and tetracyclines cause a dilution of functional oxphos complexes as a consequence of cell
division and turnover of mitochondria in non-dividing cells. This leads to a decline of $\Delta \Psi_m$, which is normally maintained by the enzyme complexes I, III and IV of the oxphos system. Hence, these antibiotics generate conditions that promote apoptosis [13,14].

The release of cytochrome $c$ from mitochondria into the cytosol is considered the primary trigger for the onset of the intrinsic apoptotic pathway [12]. When assembly of oxphos complexes is hampered by the absence of core subunits that are no longer synthesized, a surplus of free cytochrome $c$ substrate, no longer bound to oxphos complexes III or IV, may further aid leakage into the cytosol and activate the formation of the apoptosome by its effect on Apaf-1 and caspase 9. The apoptosome, in turn, activates caspase 3, the executioner of apoptosis (Figure 1).

![Figure 1](image_url)

**Figure 1**: Effect of doxycycline on metabolic and apoptotic pathways in cancer cells. Nonstandard abbreviations: $\Delta \Psi_m$, mitochondrial membrane potential; mtEGC, mitochondrial energy-generating capacity; PDC, pyruvate dehydrogenase complex; PT pore, permeability transition pore.

In recent years, several reports described effects of tetracyclines on cancer growth that were interpreted to be independent of their interference with ribosomal function [15-21] or even due to non-antibiotic properties [22,23]. However, as a direct effect on mitochondrial ribosomal function. Moreover, compared to their in vitro experiments, their in vivo experiments were performed at exceptionally low concentrations of doxycycline. The mice were treated with pellets containing 35 mg of doxycycline with a linear timed-release of 60 days. This leads to a release of ~25 μg/h. If the partition volume of the mice is arbitrarily valued at 10 ml, then this results in a concentration of 2.5 μg/ml/h. So, if this assumption is correct, the animals should have an extremely low clearance to reach the levels of $\geq 20 \mu g/ml$ at which the in vitro growth inhibition was obtained. Our experience with rats is that a constant serum level of $\sim 5 \mu g/ml$ of doxycycline requires considerably more doxycycline. We used continuous infusion with a dose of 10 mg/kg/day, a dose that was subsequently doubled weekly [24]. This doubling was necessary because an enhanced rate of doxycycline clearance was observed after prolonged treatment. In humans, we found that the permitted maximal oral dose of doxycycline, 100 mg twice a day, leads to a serum level of $\sim 5 \mu g/ml$ [25].

**Inhibition of mitochondrial protein synthesis impairs the mitochondrial energy generating capacity to drive cell proliferation**

Glucose concentrations in cancerous tissues are often 3- to 10-fold lower than in normal tissues [26,27]. A recent screen of metabolic genes identified oxphos as key metabolic pathway necessary for optimal proliferation of cancer cells under glucose limitation [28]. There is no doubt that the mitochondrial energy generating capacity is reduced by inhibition of mitochondrial protein synthesis. Therefore, we believe that tumor cell proliferation can be preferentially inhibited by interfering with the biogenesis of mitochondria. Tumor cells are more vulnerable to inhibition because their proliferation is much faster than that of most normal cells. In addition, they may have less mitochondria than normal cells, as was suggested by electron microscopy long ago [29] and has been further demonstrated by biochemistry as illustrated in Table 1. Based on cytochrome $c$ oxidase activity, tumor cell lines have a limited mitochondrial energy generating capacity compared with the type of organ from which they derived. This is in line with the original observations of Warburg [30]. Tumor cell growth arrest by actinonin and tetracyclines has been shown in numerous different in vitro and in vivo systems [6,10,14]. In experiments with nude rats transplanted with NC65 renal cancer cells, a follow-up after termination of the doxycycline treatment even showed a complete regression [8], an example of apoptosis in the strict sense of the word.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Cytochrome $c$ oxidase activity</th>
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<tr>
<td>Rat liver</td>
<td>15.6</td>
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<td>Rat Zajdela hepatoma</td>
<td>3.9</td>
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<tr>
<td>Rat kidney</td>
<td>28.5</td>
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<tr>
<td>Human NC-65 renal carcinoma</td>
<td>3.2</td>
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**Table 1**: Cytochrome $c$ oxidase activity as a measure for the mitochondrial energy-generating capacity.

Data taken from [10]. Activity is expressed as first-order rate constant per min per mg of protein. The data are the mean of $\geq 12$ measurements; S.E.M. was $\leq 10\%$ of the mean value.

**The Warburg effect**

*What are the effect and theory of Warburg?* – To obtain insights in the effect and theory of Warburg, we have lost ourselves again in some of the original publications of Warburg [30,31]. Warburg concluded that tumor cells differ from normal cells by the fact that the former can make the best of two worlds, aerobic fermentation and respiration, whereas normal cells are confined to respiration. Carbohydrate metabolism was studied in rats bearing the transplantable Jensen’s
sarcoma [30]. Glucose and lactate levels were measured in arterial and venous blood of normal organs and of the tumor in resting, narcotized animals. Normal tissues showed a moderate uptake of glucose (2–18 mg/100 ml of blood), whereas in tumor the uptake was much higher (~70 mg/100 ml of blood). Normal tissues did not excrete lactate under these experimental conditions, the tumor produced 46 mg lactate per 100 ml of blood. This high degree of fermentation by tumors became known as the Warburg effect. The Warburg theory hypothesized that tumor cells contain dysfunctional mitochondria with as a consequence that fermentation takes place even in the presence of oxygen. In the same publication, it was already stated that aerobic fermentation as well as respiration should be inhibited to kill tumor cells in vivo. Already at that time, respiration was considered crucial for the tumor. In 1956, Warburg and his group focused on ascites cells “because these, living free in the abdominal cavity, are almost pure cultures of cancer cells” [31]. This work confirmed the Warburg effect in tumor cell suspensions.

Can aerobic fermentation provide sufficient energy in cancer cells? – Tumors and cancer cells do not have responsibility for the energy devouring tasks that organs have. Being dedifferentiated, their “tasks” are restricted to growth and proliferation at a non-committal rate. Fuel is provided free of charge and cancer cells can afford to be penny-wise and pound-foolish. In times of limited energy supply, they can take a time-out and await better times for growth and proliferation. This happens when mitochondria do not function properly for whatever reason. Cancer cells can spoil lactate and disregard oxygen; the lost control on glucose uptake supports their way of life and survival. This is, in fact, what Warburg showed. Experimental evidence for the conviction that aerobic fermentation alone can keep the ATP level high comes from the work of Ramanathan and colleagues [32]. They showed for a fibroblast cell line that the ATP level can reach the original value in the course of tumorigenic conversion in the presence of oligomycin, a potent inhibitor of oxphos; however, these experiments do not permit the conclusion that cancer cell can survive independently of oxphos.

How can we unite the various observations? – The Warburg effect is irrefutable, the Warburg fact! Aerobic fermentation results in a high ATP level in the cytoplasm of cancer cells (Figure 2). That aerobic fermentation alone is sufficient to maintain this level follows from the experiments with oligomycin [32]. The question remains: enough for what? The answer is: energy generation without a major contribution from oxphos is adequate for survival but not for growth and proliferation. It appears that the advantage of the Warburg effect for cancer cells is not the dysfunction of mitochondria, as hypothesized by Warburg, but the limited demand for ATP from mitochondria for survival. This leads to a restricted ADP-ATP exchange across the mitochondrial membranes and, consequently, results in a high ATP/ADP ratio within mitochondria and a high ΔΨm. This impasse increases the apoptotic threshold and, hence, the survival of cancer cells.

What about oncogenes? – One may analyze the main feature of the Warburg effect in depth: is it that cancer cells and tumors use glucose for aerobic fermentation or that they are provided with an overdose of this suitable substrate? Are cancer cells bulimic or are they forced to accept glucose and can they only dispose of it by producing lactate? If so, the ATP production via aerobic fermentation is a side-effect that outst mitochondria from the intracellular energy market. Is there, possibly, a role or a cause related to oncogenes? This may indeed be the case. The serine/threonine kinase Akt is involved in the control of glucose import into cells. Constitutive activity of Akt has been observed as a common perturbation of malignant cells [33]. The experimental data have been interpreted to mean that the induction of the Akt oncogene is sufficiently strong to make cancer cells dependent on aerobic fermentation for continued growth and survival. This conclusion is, however, not fully corroborated by the observation that (pre) malignant cells did not proliferate in culture under comparable conditions [34].

It remains difficult to decide whether aerobic fermentation initially arises as an answer to local hypoxia and results in constitutive up-regulation or otherwise. The various possibilities for up-regulation of aerobic fermentation are interesting but the mechanism as such is less important than the actual effect itself [35].

Metabolic intervention routes
To tackle the problem of eliminating cancer cells, two main routes can be exploited: preventing the use of the surplus of glucose or trying to solve the deadlock of mitochondria. The former can be achieved with anti-metabolites, the latter by selective inhibition of mitochondrial protein synthesis, e.g. with doxycycline. The mechanism of action of anti-metabolites and doxycycline is very different: anti-metabolites affect general metabolic processes, doxycycline acts as a specific inhibitor of the expression of mitochondrial genes. Anti-metabolites act directly, doxycycline only after a lag-period. This lag-phase depends on the turnover of mitochondria and the proliferation rate of the cells. As a consequence, anti-metabolites will affect all cells and tissues, whereas doxycycline will preferentially affect cells and tissues with a high proliferation rate and relatively few mitochondria.

Inhibiting mitochondrial protein synthesis may well have indirect effects on the metabolism of glucose. Dichloroacetic acid (DCA), an inhibitor of pyruvate dehydrogenase kinase (PDK), was launched by Michelakis et al. [36] as an ideal compound to abrogate suppression of mitochondrial function and the resistance to apoptosis that characterizes cancer. Pyruvate dehydrogenase, part of the pyruvate dehydrogenase complex (PDC), is inactivated within mitochondria by phosphorylation of one of its serine residues. This reaction is catalyzed by PDK and is ATP-dependent. The reasoning of Michelakis and colleagues was that inhibition of cancer growth can be obtained by inhibiting PDK with DCA, as this would force the mitochondria to metabolize pyruvate via oxphos and, as a result, reverse the suppressed sensitivity to apoptosis. It is tempting to assume that DCA is inactive at a low mitochondrial ATP/ADP ratio. This means that the group of Michelakis pursues a goal that may also be reached by specific inhibition of mitochondrial protein synthesis.

In addition to being a main energy source, glucose also plays an important role in providing metabolites and building blocks for the synthesis of macromolecules needed for cell proliferation. Examples are NADPH and pentose phosphates. The pentose phosphate pathway (PPP) plays a crucial role in this process (Figure 2). Thiamin pyrophosphate-dependent transketolases are key enzymes in the non-oxidative part of the PPP. Transketolases are over-expressed in colon carcinomas [37]. Inhibition of transketolases with oxithiamine has been proposed as a possible agent for down-regulation of the PPP [37]; however, in vivo oxithiamine treatment may not be appropriate because thiamine (vitamin B1) is a co-enzyme of many other enzymes, e.g. PDC. Therefore, such a treatment could interfere with other essential biochemical pathways. Also here doxycycline can take over the responsibility for the inhibition.

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levels needed for the inhibition of tumor growth. Doxycycline is used in the treatment of Lyme disease at an oral dose of 100 mg/12 hours for periods of 4 weeks or longer and registered for treatment scenarios that are needed when used as anticancer drug. In the past, patients suffering from tumors of the larynx-pharynx were routinely pretreated with doxycycline to avert secondary infections. At one stage, the antibiotic of choice changed to erythromycin. As there was the impression that this change was not an improvement, a retrospective study was undertaken [39]. This revealed that some groups of patients had been better off with doxycycline. On basis of the current state of knowledge, a serious examination of the potential benefit of doxycycline on its own or in combination therapy should be considered.

**Conclusions**

The Warburg effect is based on the infinite uptake of glucose (Akt is constitutively present) and cannot be reversed. Due to the Warburg effect, the demand for ATP from mitochondria is limited in cancer cells, leading to a restricted ADP-ATP exchange across the mitochondrial membranes. This results in a high ATP/ADP ratio within mitochondria and a high ΔΨm. In turn, this makes cancer cells more resistant to apoptosis. The Warburg effect provides sufficient energy for survival but not for growth and proliferation. The Warburg effect should not be interpreted to mean that the mitochondria in cancer cells are crippled. In fact, mitochondria are on stand-by and only called upon when there is a high demand for energy to support biomass increase for proliferation. Upon inhibition of mtDNA expression, cells are arrested in G1, prior to the biosynthesis and energy peak of the cell cycle. Inhibition of expression of mtDNA offers an attractive target to manipulate the mitocytosplasmic energy balance because mtDNA expression can be specifically inhibited by antibacterial drugs that inhibit mitochondrial protein synthesis or maturation. Tetracyclines, especially doxycycline, have ideal pharmacokinetic properties and have been shown to inhibit proliferation of a large variety of cancers. The effect of these antibacterial drugs is not instantaneous but will take effect after one or more rounds of cell division. Taken together, the available data justify further investigations of the use of doxycycline in cancer chemotherapy.

**Acknowledgement**

Dedicated to our colleague Dr. Coby van den Bogert who died too young.

**References**


