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MINI REVIEW

Hypoxia, glucose metabolism and the Warburg's effect

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Abstract As described by Warburg more than 50 years ago, tumour cells maintain a high glycolytic rate even in conditions of adequate oxygen supply. However, most of tumours are subjected to hypoxic conditions due to the abnormal vasculature that supply them with oxygen and nutrients. Thus, glycolysis is essential for tumour survival and spread. A key step in controlling glycolytic rate is the conversion of fructose-6-P to fructose-1,6-P_2 by 6-phospho-fructo-1-kinase (PFK-1). The activity of PFK-1 is allosterically controlled by fructose-2,6-P_2, the product of the enzymatic activity of a dual kinase/phosphatase family of enzymes (PFKFB1-4) that are increased in a significant number of tumour types. In turn, these enzymes are induced by hypoxia through the activation of the HIF-1 complex (hypoxia-inducible complex-1), a transcriptional activator that controls the expression of most of hypoxia-regulated genes. HIF-1 complex is overexpressed in a variety of tumours and its expression appears to correlate with poor prognosis and responses to chemo or radiotherapy. Thus, targeting PFKFB enzymes, either directly or through inhibition of HIF-1, appears as a promising approach for the treatment of certain tumours.

Keywords Hypoxia · Glucose metabolism · Warburg's effect

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Introduction

Some of the earliest modern studies of cancer have observed abnormalities in tumour metabolism. In pioneering studies in the 1920s, Otto Warburg observed that cancers possessed a remarkable ability to sustain high rates of anaerobic-like glycolysis even in the presence of oxygen (Warburg 1956). Anaerobic glycolysis utilizes glucose to produce lactate, while aerobic glycolysis (respiration) produces pyruvate, which enters the tricarboxylic acid cycle; ultimately, the latter produces energy via oxidative phosphorylation. An essential thermodynamic trade-off exists between these two pathways with respect to rate (moles of ATP per unit time) and yield (moles of ATP per mole substrate) with fermentation proceeding at a rate some 100 times faster than respiration, which in turn yields roughly 18-fold more ATP per mole glucose. Population biology modelling demonstrates how organisms utilize the intrinsic trade-off between these two pathways to maximal effect. Cells with a higher rate but lower yield of ATP production may gain a selective advantage when competing for shared energy resources (Pfeiffer et al. 2001).

There are numerous molecular modulators of glycolytic flux, the most famous of which was discovered in 1860 by Louis Pasteur (Pasteur 1861). Pasteur showed that oxygen inhibits fermentation and that glucose consumption is inversely proportional to the oxygen availability (Pasteur's Effect). It is now clear that the allosteric properties of 6-phosphofructo-1-kinase (PFK-1) can account for most aspects of the Pasteur's effect (Krebs 1972). Conversely, many tumours have high rates of glycolysis regardless of whether their supply of oxygen is appropriate (Warburg's effect). These tumours depend largely on the glycolytic pathway for generation of ATP to meet most of their energy needs. Warburg attributed this metabolic alteration to mitochondrial "respiration injury" and considered this as the most fundamental metabolic alteration in malignant transformation or "the origin of cancer cells" (Warburg 1956). This hypothesis was neglected because some tumours do not have defects in respiration and, also, respiration exerts a regulatory effect upon glycolysis (Chance 2005). Although some cancer cells do not have high glycolytic activity (Zu and Guppy 2004), the Warburg effect has been consistently observed in a wide spectrum of human cancers and it constitutes the physiological basis for the use of PET scans in clinical oncology. The underlying biochemical and molecular mechanisms of the Warburg's effect are probably multiple and include mitochondrial malfunction (Wallace 2005), oncogenic alterations (Dang and Semenza 1999), as well as adaptive responses to the tumour microenvironment (Gatenby and Gillies 2004).

Hypoxia and the glycolytic phenotype

In physiological conditions, the cellular energy metabolism is based, preferentially, on oxidative phosphorylation, which is more efficient than glycolysis. Mitochondria consume O_2 and burn metabolites to provide chemical power to cell, and the availability of nutrients and oxygen are important constraints on the growth of cells and tissues. During hypoxia, cells shift to a primarily glycolytic phenotype for their energetic needs. In cancer cells, the factors responsible for the Warburg effect compromise the ability of cells to generate ATP via mitochondrial respiration, which triggers alternative metabolic pathways by, among other processes, preferential use of the glycolytic pathway (Warburg 1956; Wallace 2005). The high glycolytic rate allows the cells to balance their energy demands and supply the anabolic precursors for de novo nucleotide and lipid synthesis (Bui and Thompson 2006). Furthermore, the net result of glycolytic flux to pyruvate/lactate decreases intra- and extracellular pH, causing apoptosis in normal cells that express functional p53 (Williams et al. 1999). This strategy is considered to provide a growth advantage for the tumour cell, although it renders cells highly dependent on substrate availability for survival (Gatenby and Gillies 2004).

One key mediator of the hypoxic response in animal cells is the hypoxia-inducible transcription factor (HIF-1) complex, in which the α-subunit is highly susceptible to oxygen-dependent degradation. HIF has been frequently found to be highly expressed in human cancers and HIF-1α stabilization induces the expression of a set of genes involved in erythropoiesis, angiogenesis and anaerobic metabolism (Caro 2001; Maxwell et al. 2001; Brahimi-Horn and Pouyssegur 2006; Semenza 2006). Amongst those genes turn-on by hypoxia are the glucose transporters (Glut-1 and 3) and genes encoding glycolytic enzymes such as aldolase, enolase or lactate dehydrogenase (LDH-A), and the glycolytic regulatory bifunctional enzymes 6-phospho-2-kinase/fructose2, 6-biphosphatase (PFKFB1-4) (Dang and Semenza 1999; Minchenko et al. 2002; Obach et al. 2004). All these glycolytic genes are stimulated by hypoxia through hypoxia-response elements (HRE) in their promoters. Functional HRE's consist of a pair of contiguous transcription factor binding sites at least one of which contains the core sequence 5*-RCGTG-3* that is recognized by HIF-1 (Caro 2001; Maxwell et al. 2001). Factors that recognize the two sites may function cooperatively either at the level of DNA binding or transactivation. The HIF-1 complex is a heterodimeric complex composed by two subunits; HIF1β, also known as ARNT, that is constitutively expressed and HIF1α, whose levels are dependent on the oxygen concentration in tissues. Both proteins belong to the helix-loop-helix class of transcription factors that contain a PAS domain. HIF-1 complex formation is a multistep process regulated primarily by the levels of the HIF1α subunit (Semenza 2002). In well oxygenated cells HIF1α has an extremely short half life due to its rapid degradation by the ubiquitin-proteasome system (Huang et al. 1996; Salceda and Caro 1997). Thus, in normoxic cells, HIF1α steady state levels are usually undetectable and they rapidly increase during exposure to hypoxia. The normoxic degradation of HIF1α depends on the presence of an aminoacid sequence known as the oxygen-dependent degradation domain (ODDD) located at the centre of the protein. The ODDD contains two prolyl-residues (Pro402 and Pro564) that, in the presence of oxygen, are hydroxylated by specific enzymes known as HIF-prolyl hydroxylases (HIF-PHD's) (Gleadle and Ratcliffe 1998). Once hydroxylated, HIF1α is recognized by the von Hippel-Lindau (VHL) protein that acts as an ubiquitin- ligase (E3) (Pugh and Ratcliffe 2003; Mole et al. 2001; Kaelin 2005). Ubiquitylated HIF1α is then rapidly degraded by the 26S proteasome (Fig. 1). HIF-PHD's are iron- and oxoglutarate-dependent enzymes and their activity is greatly inhibited by iron-chelators and by increases in oxoglutarate levels (Metzen and Ratcliffe 2004; Schofield and Ratcliffe 2005). VHL is a critical regulator of HIF1α survival and VHL-

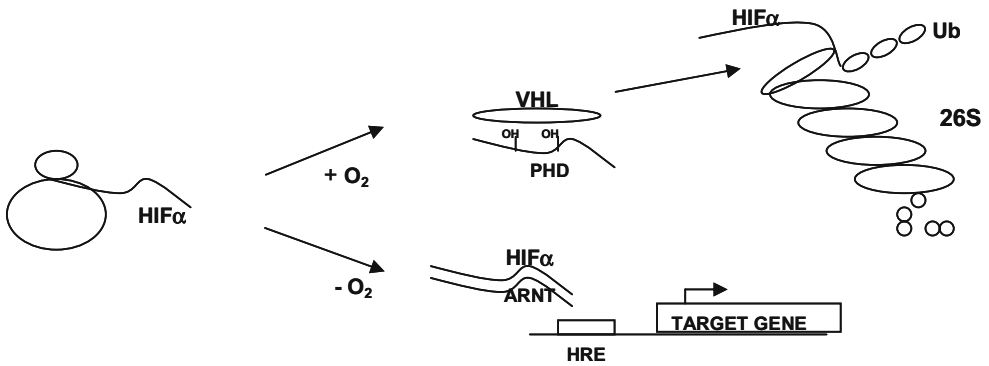

Fig. 1 Schematic representation of the activation of the HIF-1 complex by hypoxia. During normoxia ($+O_2$), HIF1α protein is hydroxylated by prolylhydroxylase enzymes (PHD) enhancing its interaction with VHL that acts as an ubiquitin-ligase, promoting its degradation by the 20S proteasome. In hypoxia ($-O_2$), HIF1α survives, translocates to the nuclei where it interacts with ARNT to form an active HIF-1 complex that binds HRE sequences and transactivates downstream genes

deficient cells express high levels of HIF-1 and HIF-regulated genes, even in normoxic conditions (Kaelin 2005).

Activation of glycolysis during hypoxia produces an increase in glycolytic ATP production as mitochondria become starved for O_2, the substrate for oxidative phosphorylation (Esteban and Maxwell 2005). Thus, mitochondrial respiration passively decreases due to O_2 depletion in hypoxic tissues. However, it has been demonstrated recently that this critical metabolic adaptation to hypoxia is more complex and includes an active suppression of mitochondrial pyruvate catabolism and O_2 consumption that is also mediated by HIF-1 (Kim et al. 2006a,b; Papandreou et al. 2006). HIF-1 suppresses both the TCA cycle and respiration by inducing pyruvate-dehydrogenase kinase 1 (PDK1), which inhibits pyruvate dehydrogenase activity by phosphorylating its E1 subunit (Fig. 2). By doing so, pyruvate entry into the TCA cycle is limited and hypoxic cells accumulate pyruvate, which is then converted into lactate via LDH. Lactate in turn is released into the extracellular space, regenerating NAD+ for continued glycolysis by O_2-starved cells. This HIF-1-dependent block to mitochondrial O_2 consumption promotes cell survival, especially when O_2 deprivation is severe and prolonged. Inhibition of the pyruvate dehydrogenase complex by PDK1 attenuates not only mitochondrial respiration but also the production of mitochondrial reactive oxygen species in hypoxic cells, thus promoting cell survival (Kim et al. 2006a,b; Papandreou et al. 2006).

The rate of glycolytic flux is controlled at different levels and by different mechanisms: substrate availability, enzyme concentrations, allosteric effectors and covalent modification on regulatory enzymes. One of the critically modulated steps is that catalyzed by PFK-1, with fructose-2, 6-bisphosphate (Fru-2,6-P_2) being its most powerful allosteric activator (Van Schaftingen 1987; Okar and Lange 1999; Rider et al. 2004). Fru-2,6-P_2 relieves ATP inhibition and acts synergistically with AMP, and in addition it inhibits fructose 1,6-bisphosphatase (Van Schaftingen 1987). These properties confer to this metabolite a key role in the control of fructose 6-P/fructose 1,6-P_2 substrate pathway. It has been found that PFK-1 expressed in human lymphomas and gliomas is less sensitive to citrate inhibition and more sensitive to Fru-2,6-P_2 stimulation (Colomer et al. 1987; Staal et al. 1987). In addition, PFK-1 activity is increased in transformed cell lines and tumours, and activated by *ras* and *src* oncogenes (Bosca et al. 1985; Hennipman et al. 1988; Kole et al. 1991). Likewise, Fru-2, 6-P_2 is also increased in transformed cells and tumors (Hue and Rousseau 1993).

The steady-state concentration of Fru-2,6-P_2 depends on the activity of different homodimeric bifunctional enzymes designed as 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2) that catalyze the synthesis and degradation of Fru-2,6-P_2 and hence critically regulates carbohydrate metabolism (Van Schaftingen 1987; Okar and Lange 1999; Rider et al. 2004). Four independent genes (PFKFB1–4) code for the different isoforms of the PFK-2 family (Okar et al. 2001). These isoforms show differences in their tissue distribution and kinetic properties in response to allosteric effectors, hormonal, and growth factor signals (Okar et al. 2001). The expression of these genes is dependent on tissue and on development stage (Goren et al. 2000). Importantly, tissue- and cell-specific isoenzymes are not totally exclusive and several cells express more than one isoenzyme (Minchenko et al. 2005a,b; Calvo et al. 2006; Telang et al. 2006). This pattern of expression suggests that each isoenzyme plays a key role under different physiological conditions or in response to different stimuli.

One of the PFK-2 family genes (PFKFB3), firstly isolated from brain (Ventura et al. 1995) and from other different cells and tissues (Sakai et al. 1996; Hamilton et al. 1997; Manzano et al. 1998; Chesney et al. 1999), expresses an isoenzyme which can be induced by different factors (iPFK-2) and has the highest kinase/bisphosphatase ratio,

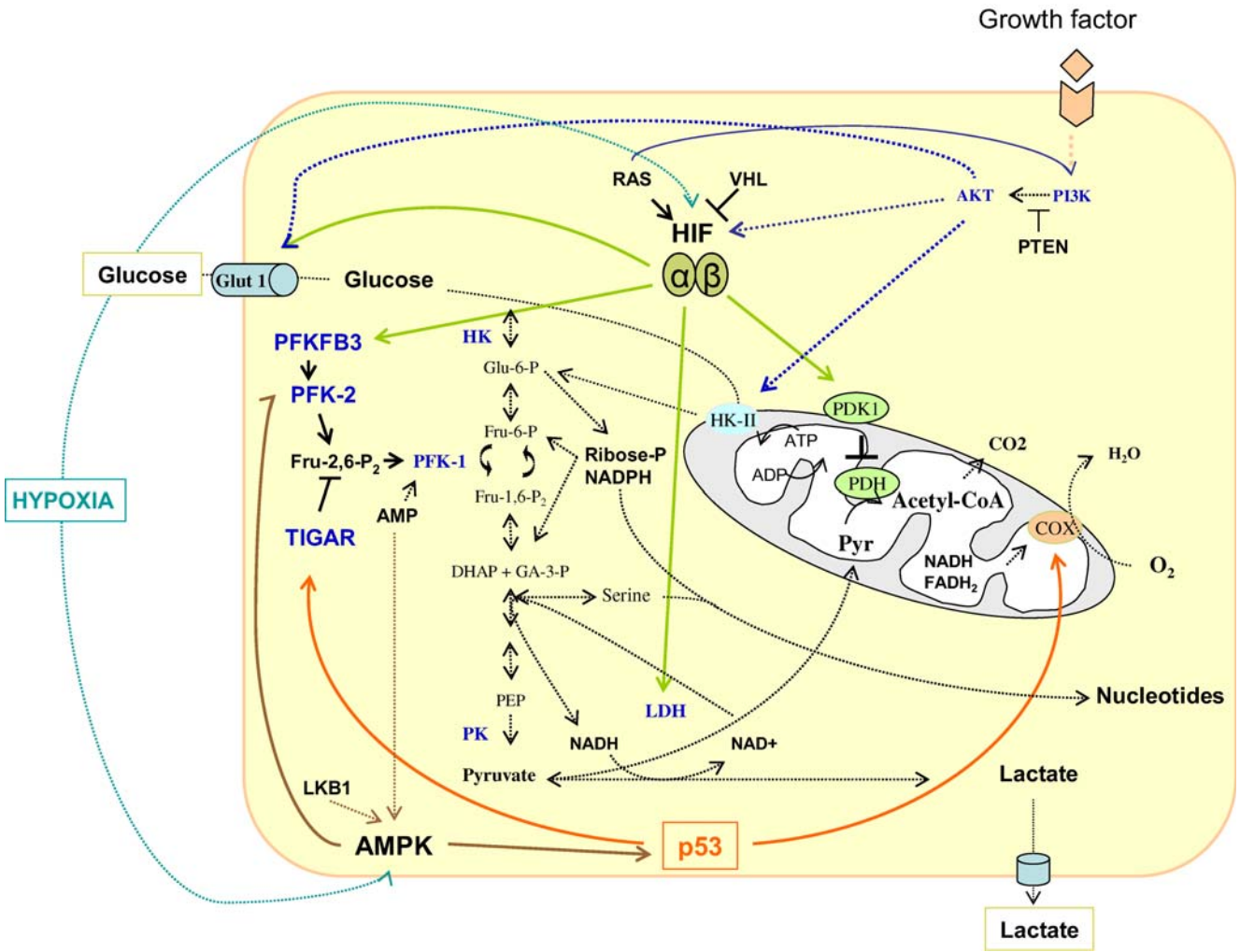

Fig. 2 Molecular mechanisms of the Warburg effect. The Warburg effect describes the enhanced conversion of glucose to lactate by tumor cells, even in presence of adequate oxygen concentrations. Activation of AKT results in increase Glut1 transporter, binding of HK-II to mitochondria and enhanced glycolytic flux. Hypoxia produces stabilization of HIF-1, increased also by activation of *ras* and loss of VHL, which transactivates glycolytic genes (PFKFB3, LDH) as well as PDK1, which in turn inhibits PDH that catalyzes the conversion of pyruvate to acetyl CoA. Inhibition of PDH blocks the entry of pyruvate to Krebs cycle. Additional PFK-2 enzyme activation by AMP-dependent protein kinase (AMPK) reinforces the glycolytic flux. p53 stimulates respiration through induction of SCO2, component of cytochrome c oxidase (COX), and decreases glycolysis by inducing TIGAR, increasing this effect the flux of the pentose phosphate shunt to produce NADPH and ribose-5P

favouring net synthesis of Fru-2,6-P_2 (Sakakibara et al. 1997). The PFKFB3 gene is distinguished by the presence of multiple copies of the AUUUA sequence in the 3'UTR. The AUUUA motif is typical of proto-oncogenes and proinflammatory cytokines and confers instability and enhanced translational activity (Chesney et al. 1999). The iPFK-2 isozyme is degraded through the ubiquitin-proteasome proteolytic pathway (Riera et al. 2003). Its expression is induced in response to different stimuli such as progesterone (Hamilton et al. 1997), serum, insulin (Riera et al. 2002) or proinflamatory molecules (Chesney et al. 1999). In addition, iPFK-2 isoenzyme is highly induced by hypoxia (Minchenko et al. 2002), through HIF interaction with the consensus HRE sites in its promoter region (Obach et al. 2004). This induction could be replicated by the use of inhibitors of the prolyl-hydroxylase enzymes responsible for the von Hippel Lindau (VHL)-dependent destabilization and tagging of HIF-1α. The dependence of the PFKFB3 gene expression on HIF-1 is confirmed by its overexpression in VHL-deficient cells and by the lack of hypoxic induction in mouse embryonic fibroblasts conditionally nullizygous for HIF-1α (Minchenko et al. 2002; Obach et al. 2004). Other PFK-2 isoenzymes, more remarkably, the testicular isoform (PFKFB4) can also be modulated by hypoxia but to a lesser extent (Minchenko et al. 2003, 2005a; Gomez et al. 2005).

During times of hypoxia, nutrient or environmental stress, mammalian cells sense AMP-activated protein kinase (AMPK), a highly conserved sensor of cellular energy status found in all eukaryotic cells (Hardie 2004).

AMPK is activated by stimuli that increase the cellular AMP/ATP ratio. Essential to activation of AMPK is its phosphorylation by an upstream kinase, the LKB1 serine/threonine kinase, the gene inactivated in the Peutz-Jeghers familial cancer syndrome (Hong et al. 2003). Activated AMPK phosphorylates diverse targets, including many that are directly involved in controlling cellular energy metabolism (Hardie 2004) and cell-cycle arrest through p53 activation (Jones et al. 2005). Activation of AMPK by hypoxia results also in the phosphorylation of iPFK-2, increasing its Vmax and the subsequent increase in Fru-2, 6-P_2 content and glycolysis (Marsin et al. 2002). Moreover, highly phosphorylated iPFK-2 protein has been found in human tumor cells (Bando et al. 2005). Activation of AMPK in hypoxic or ischemic microenvironments may be critical for cell survival and thus would represent a protective mechanism for metabolically depressed or ATP-deficient cells. These findings imply that HIF-1 and AMPK are components of a concerted cellular response to maintain energy homeostasis in low-oxygen or ischemic-tissue microenvironments.

The specific role of induction or activation of PFK-2 by hypoxia must be related with its key function on PFK-1 stimulation. PFK-1 is mainly inactive in the cell in the absence of allosteric modulators, and the main role of Fru-2,6-P_2 is to relieve its ATP inhibition, allowing glycolysis to proceed (Van Schaftingen 1987). The PFKFB3 gene product is present in proliferating tissues (Sakai et al. 1996; Manzano et al. 1998; Goren et al. 2000) and transformed cells (Hamilton et al. 1997; Chesney et al. 1999; Riera et al. 2002; Minchenko et al. 2002; Obach et al. 2004; Calvo et al. 2006) and various tumors (Atsumi et al. 2002). The high kinase/bisphosphatase activity ratio of this isozyme can explain the high Fru-2,6-P_2 found in the cells where it is present, which in turn sustains high glycolytic rates. Similarly, the PFKFB4 isoform has been found to be increased in several tumours, including those of the mammary gland, and it is likely to be involved in the regulation of glycolysis in those tumours (Minchenko et al. 2005a,b).

Oncogenes and the Warburg's effect

It is clear that not all areas of a tumour that are highly glycolytic are necessarily hypoxic (Rajendran et al. 2004). Hence, there must be other regulatory mechanisms inducing the glycolytic phenotype. Many tumour cells growing under conditions of normal oxygen tension also show elevated glycolytic rates that correlate with the increased expression of glycolytic enzymes and glucose transporters (Dang and Semenza 1999). This could be due to the stabilization of HIF-1α by factors other than hypoxia, for example the inactivation of VHL (Kaelin 2005; Kim and Kaelin 2006) or the activation of HIF by growth factors or different oncogenes (Semenza 2002; Thornton et al. 2000; Zhou and Brune 2006). It has been shown that oncogenes per se can also activate glycolysis. *Ras* oncogenes can enhance glucose transport and glycolysis in transformed cells (Mazurek et al. 2001). One of the targets has been shown to be PFK-1 (Kole et al. 1991). In addition, the expression of the iPFK2 has been recently shown to be selectively necessary for the control of glycolytic flux in cells transformed with *ras* (Telang et al. 2006). Furthermore, it is interesting that most glycolytic enzymes contain evolutionarily conserved consensus Myc-binding sites or E-boxes among their regulatory DNA sequences. Myc oncogene binds to these promoters and transactivates them under normoxic conditions (Kim and Dang 2006). Akt, independent of HIF-1, can also activate glycolysis without an increase in oxidative phosphorylation (Hatzivassiliou et al. 2005). Akt induces glucose transporters, and signals a glycolytic enzyme, hexokinase (HK-II), to bind tightly to the mitochondria membrane, having preferential access to the ATP generated and removing the inhibitory effect of glucose-6-phosphate (Bustamante and Pedersen 1977; Mathupala et al. 2006). There is a general inverse correlation between mitochondrial hexokinase association and apoptotic susceptibility (Hatzivassiliou et al. 2005; Mathupala et al. 2006). Akt has also been found to activate the heart PFK-2 isoenzyme (Pozuelo Rubio et al. 2003). The regulation of HIF-1α protein synthesis and activity by PI3K-AKT pathway is at present controversial (Hatzivassiliou et al. 2005; Kim and Dang 2006).

Another important transcriptional regulator of tumor metabolism is p53. In response to cellular stressors such as oncogene activation or DNA damage, wild-type p53 becomes stabilized and switches on the expression of target genes. These target genes drive a variety of cellular responses to stress including DNA repair, cell-cycle arrest, senescence, and apoptosis (Vousden and Lu 2002). Recently, a new dependent p53 gene, named TIGAR (TP53-induced glycolysis and apoptosis regulator), has been described (Bensaad et al. 2006). TIGAR is a novel PFK-2 isoenzyme which shares functional sequence similarities with the bisphosphatase domain of the bifunctional enzyme but lacks the kinase domain. TIGAR causes a decline in Fru-2, 6-P_2 levels and thereby blocks glycolysis at this step, directing the pathway into the pentose phosphate shunt to produce NADPH (Fig. 2). One consequence of the pentose phosphate shunt and increased NADPH generation is an increase in glutathione (GSH) levels, which promote the scavenging of reactive oxygen species (ROS). Expression of TIGAR protected cells from ROS and moderately protected cells from DNA damage-induced apoptosis. Other recent findings support a model in which p53 target genes

preserve genomic stability by decreasing the oxidation of DNA (Sablina et al. 2005). The function of TIGAR may be to direct glucose away from energy production and toward the synthesis of nucleotides and other products that might be important for the repair of DNA lesions. Furthermore, a recent study (Matoba et al. 2006) has shown that the p53 protein induces production of a copper transporter, SCO2 (synthesis of cytochrome *c*-oxidase-2), which participates in the assembly of cytochrome *c*-oxidase (COX) in mitochondria. Cells lacking p53 have diminished oxygen consumption, which could be restored by ectopic expression of SCO2. Under conditions where mitochondrial oxygen consumption becomes faulty, ROS may be generated resulting in activation of p53 to halt glycolysis (thus restricting the fuel to drive transfer of electrons to molecular oxygen). The action of p53 via TIGAR also provides NADPH to increase GSH levels that are needed to scavenge ROS; this feeds back to stop the damage signals, presumably while DNA repair intermediates are maintained. Meanwhile, during oncogenesis, all of these steps are bypassed because in cells that accumulate defects in the p53 pathway, glycolysis proceeds at full steam and the normal restraints on tumour growth are lost.

Tumours make energy starts with upstream gene mutations that activate Akt and end with cancer cells continuously consuming glucose. Furthermore, cancer cells rely almost completely on glycolysis and largely shut down respiration, as Warburg originally reported. Cancer cells could benefit from glycolysis in many ways. A boost in glycolysis, added to respiration, generates energy at a rate higher than in normal cells that overwhelmingly rely on respiration (Wallace 2005). Mitochondrial hexokinase II association, induced by AKT, is thought to prevent apoptosis (Hatzivassiliou et al. 2005; Mathupala et al. 2006). Exposure to hypoxia either induces or selects for cells with the glycolytic phenotype and this in turn produces local acidosis (Gatenby and Gillies 2004). Acidification of the micro- environment would provide a selective growth advantage for cells that have lost wild type p53 function, leading to clonal expansion of aberrant cell populations (Gatenby and Gillies 2004). Since the glycolytic cancer cell is constantly slurping up nutrients, whereas a normal cell typically needs outside signals for permission to do this, such energy independence empowers the cancer cell to grow (Fantin et al. 2006). Other potential benefits are that glycolysis leads directly to HIF-1 activation, which further boosts metabolism, and also stimulates angiogenesis and invasiveness. Furthermore, in cases in were respiration is impaired, shutting it down protects cancer cells from mitochondria oxidative damage that occurs when cellular respiration functions abnormally under hypoxic conditions. The glycolytic shift is not absolutely required for transformation, but it gives cell-autonomous metabolism, tolerance to acidosis and a higher proliferative and invasive potential.

Implications for treatment

The dependence of cancer cells on survival mechanisms that are coupled to energy mechanisms suggests several potential therapeutic avenues. Recent studies have provided evidence indicating that HIF-1 mediates resistance to chemotherapy and radiation (Semenza 2006). Inhibition of HIF-1 activity could therefore represent an important component of anticancer therapy (Maxwell 2005). Transformed cells may be particularly sensitive to inhibition of glycolytic flux when oxygen and glucose limitations are imposed by surrounding cells. This has been true in experiments using glycolytic inhibitors (Ko et al. 2004; Pelicano et al. 2006) or inhibiting the expression of different glycolytic enzymes (Engel et al. 2004; Kondoh et al. 2005; Fantin et al. 2006). Recently, it has been shown that siRNA against iPFK-2 decreased cell proliferation and greatly inhibited anchorage-independent growth (Calvo et al. 2006). The recent report of the X-ray crystal structure of iPFK-2 presents a great opportunity for the development of cancer therapeutic molecules with high specificity (Kim et al. 2006a,b).

The finding that PFKFB3-4 genes are overexpressed in different tumours, activated by hypoxia and/or oncogenes, indicates that they have an essential role in the glycolytic phenotype of cancer cells, facilitating the adaptation and survival of proliferating and tumour cells in their hypoxic microenvironment. Suppression of glycolysis may be an effective therapeutic for cancer therapies and, accordingly, the inhibition of PFKFB3-4 expression could be one of the targets of such an approach.

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