The emerging role of fumarate as an oncometabolite

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The drive to understand how altered cellular metabolism and cancer are linked has caused a paradigm shift in the focus of cancer research. The discovery of a mutated metabolic enzyme, isocitrate dehydrogenase 1, that leads to accumulation of the oncometabolite 2-hydroxylutarate, provided significant direct evidence that dysfunctional metabolism plays an important role in oncogenesis. Striking parallels exist with the Krebs cycle enzyme fumarate hydratase (FH), a tumor suppressor, whose mutation is associated with the development of leiomyomata, renal cysts, and tumors. Loss of FH enzymatic activity results in accumulation of intracellular fumarate which has been proposed to act as a competitive inhibitor of 2-oxoglutarate-dependent oxygenases including the hypoxia-inducible factor (HIF) hydroxylases, thus activating oncogenic HIF pathways. Interestingly, our studies have questioned the role of HIF and have highlighted other candidate mechanisms, in particular the non-enzymatic modification of cysteine residues (succination) that could lead to disruption or loss of protein functions, dysfunctional cell metabolism and cell signaling. Here, we discuss the evidence for proposing fumarate as an oncometabolite.

Keywords: fumarate, oncometabolite, succination, dysregulated metabolism, mitochondrial dysfunction

THE LINK BETWEEN DYSREGULATED METABOLISM AND CANCER

Cancer cells exhibit characteristic "hallmarks" of malignancy including increased proliferation, survival, and in particular dysregulated metabolism (Hanahan and Weinberg, 2011). There is abundant evidence showing that cancer cells produce energy through a high rate of glycolysis in the cytoplasm, in marked contrast to the process in most normal cells, which employ a relatively low rate of glycolysis followed by oxidation of pyruvate in the mitochondria (Kim and Dang, 2006). Although Otto Warburg postulated that this switch in cellular metabolism was the fundamental cause of cancer, most cancer research since has focused on mutations in, and roles of, oncogenes and tumor suppressors in the onset and progression of cancers (Warburg et al., 1927; Warburg, 1956; Semenza et al., 2001; Vander Heiden et al., 2009). The development and application of highly sensitive new technologies such as mass spectrometry and nuclear magnetic resonance combined with metabolic labeling and profiling have increased our understanding of the complexities of normal and dysregulated cellular metabolism, particularly when linked with powerful computing programs that allow for the integration and interrogation of data (Tomita and Kami, 2012). Furthermore, cancer associated mutations have been identified in genes of known metabolic function; namely isocitrate dehydrogenase 1 and 2 (IDH1 and 2), succinate dehydrogenase (SDH) and fumarate hydratase (FH; Semenza, 2011). Consequently, there has been renewed interest in Warburg’s hypothesis and the link between dysregulated metabolism and cancer.

WHAT IS AN ONCOMETABOLITE?

The term oncometabolite has only recently been coined and assigned with confidence to (R)-2-hydroxylutarate ((R)-2HG), the reduced form of 2-oxoglutarate (2OG). (R)-2HG is a byproduct produced by gain-of-function mutations of IDH1 and IDH2, which normally catalyze the reversible NADP+-dependent oxidative-decarboxylation of isocitrate to produce 2OG in the cytoplasm and mitochondria, respectively (Leonardi et al., 2012). IDH mutations have been found in 75% of low grade gliomas and secondary glioblastoma multiforme and approximately 20% of acute myeloid leukemia (Parsons et al., 2008; Mardis et al., 2009; Yan et al., 2009). 2HG acts as a competitive inhibitor to multiple 2OG utilizing 2-oxogénases, including prolyl hydroxylases (PHDs), histone demethylases, and the TET family of 5-methylcytosine (5mC) hydroxylases (Chowdhury et al., 2011; Xu et al., 2011). In gliomas, (R)-2HG accumulation caused by oncogenic IDH mutations enhances DNA methylation and epigenetic remodeling, which stalls cell differentiation and thereby primes cells for malignancy (Figureau et al., 2010; Ward et al., 2010; Lu et al., 2012).

How should we define an oncometabolite? Using (R)-2HG as an example, one could propose that an oncometabolite is a small molecule component (or enantiomer) of normal metabolism whose accumulation causes metabolic dysregulation and consequently primes cells allowing future progression to cancer. There are likely to be numerous and complex interacting steps in this process including inhibition, disruption or activation of pathways each of which will require detailed investigation. Nevertheless, the concept of oncometabolites is novel and exciting and...
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deficient human tumors (O’Flaherty et al., 2010; Adam et al., whole animal physiology with successful extrapolation into FH-the complex consequences of FH loss for cellular, tissue and the mitochondria and the cytoplasm and to unravel some of have been used to investigate the importance of FH in both Fh1-deficient MEFs in which there is stable re-expression of either FH) knockout mouse model (Pollard et al., 2007) and a panel tion we have used a conditional such as fumarate accumulation (Frezza et al., 2011a). These have been used to investigate the importance of FH in both the mitochondria and the cytoplasm and to unravel some of the complex consequences of FH loss for cellular, tissue and whole animal physiology with successful extrapolation into FH-deficient human tumors (O’Flaherty et al., 2010; Adam et al., 2011). Immunofluorescence studies with the MEFs described above have demonstrated that Fh1 loss results in a striking change in the morphology of mitochondria, which become much enlarged (O’Flaherty et al., 2010). This phenotype reinforces the observation that mitochondrial dysfuction is associated with FH deficiency; but the precise reasons for this and the consequences for the mitochondria and the cell remain to be determined. It could be postulated that disruption to the Krebs cycle leads to alterations in mitochondrial membrane potential and permeability of the outer membrane and increased autophagy; all aspects of cell biology and physiology that can, and should, be investigated.

FUMARATE ACCUMULATION – A CONSEQUENCE OF FH INACTIVATION

Fumarate hydratase-deficient cells and tumors have been shown to accumulate fumarate to very high levels with multiple consequences including the activation of oncogenic pathways (Isaacs et al., 2005; Pollard et al., 2005). In Fh1-deficient MEFs the level of fumarate is approximately 8–10 fmol/cell as measured by 1H magnetic resonance spectroscopy metabolite analysis and no fumarate can be detected by this technique in either wild-type MEFs or Fh1−/−/FH MEFs (O’Flaherty et al., 2010). Perhaps surprisingly only very low levels (approximately 1 fmol/cell) can be detected in Fh1-deficient MEFs complemented with extramitochondrial FH (Fh1−/−/+HAMTS), although the defect in aerobic metabolism is not corrected (O’Flaherty et al., 2010). Currently, we are undertaking metabolomic analyses to confirm these observations in MEFs by alternative techniques (capillary electrophoresis time-of-flight mass spectrometry; Soga et al., 2003, 2006) and to extend the studies to mouse and human tissues lacking FH. It would be interesting to determine the relative levels of fumarate under a variety of physiological conditions in different cellular compartments; mitochondrial versus cytoplasm – especially since cytoplasmic “rescue” effects such a dramatic reduction in the overall cellular fumarate levels (O’Flaherty et al., 2010) and in the nucleus, given the proposed role for FH in the DNA damage response in yeast (Huyghe et al., 2010).

COMPETITIVE INHIBITION OF 2-OXOGLUTARATE-DEPENDENT OXYGENASES

Others had postulated previously that FH-associated tumorigenesis might be driven by the upregulation of a number of oncogenic pathways by hypoxia inducible factor (HIF; Gottlieb and Tomlinson, 2003). Indeed, it has been shown that fumarate competitively inhibits 2OG-dependent oxygenases, particularly the HIF PHDs, thus mimicking hypoxia (psuedohypoxia), stabilizing the HIF complex and potentially activating its oncogenic target genes (Isaacs et al., 2005). Hypoxia inducible factor is stabilized in human tumors in HLRCC, in Fh1-deficient MEFs and in the hyperplastic renal cysts that develop in mice following targeted inactivation of Fh1. Gene expression analysis in all these tissues revealed strong signatures of HIF activation (Isaacs et al., 2005; Pollard et al., 2005, 2007; Ashrafian et al., 2010). Furthermore, both succinate and fumarate inhibit PHD enzymatic activities in vitro and cell-permeable esters of 2OG reactivate the enzymatic activity of the PHDs and alleviate the pseudohypoxia caused by succinate or fumarate accumulation (Hewison et al., 2007; Mackenzie et al., 2007). However, using a mouse model in which Fh1 inactivation in renal tubular cells was combined with inactivation of Hif-1a, Hif-2a, or both Hif-1α isoforms; hyperplastic cyst formation was shown to be Hif independent (and separately Phd independent). Indeed combined inactivation of Fh1 and Hif-1α greatly exacerbated the cystic hyperplasia (Adam et al., 2011). While this suggests that the effect of HIF may be discounted in the early events of fumarate-mediated oncogenesis it neither precludes a role in tumorigenesis.
FIGURE 1 | Consequences of elevated cellular fumarate. Loss of fumarate hydratase enzyme activity results in intracellular accumulation of fumarate with multiple diverse consequences. However, it remains to be determined whether some, or all of these, or indeed other as yet uncovered pathways, lead directly to oncogenesis. Dysregulated metabolism possibly linked to reductive carboxylation may both result from elevated fumarate and is certainly a cause of the elevated fumarate. Mitochondrial dysfunction is a feature of both altered metabolism and possibly high fumarate levels, but whether it is a contributing factor in oncogenesis needs to be determined and if autophagy leads to increased availability of nutrients for the cell. Fumarate has been shown to act as a competitive inhibitor of members of the 2-oxoglutarate-dependent oxygenase superfamily including the histone demethylase enzymes (HDMs), TET proteins and hypoxia-inducible factor (HIF) hydroxylases, thus activating oncogenic HIF pathways. However, further investigation is required to ascertain whether fumarate initiates oncogenesis via all, or any, of these routes. Succination of cysteine residues that could lead to disruption or loss of protein functions, dysfunctional cell metabolism and cell signaling offers a novel and promising route to link fumarate and oncogenesis directly. The benefits of fumarate proposed in activating a DNA damage response need to be addressed further, while the cytoprotective role proposed for fumarate in cardiac cells by diverting amino acids into the Krebs cycle and activating the Nrf2 antioxidant pathway suggests that different cell types may have different response strategies.

for long-term stabilization of HIF nor its consequent activation of multiple oncogenic pathways.

This is by no means an end to the story as recent evidence has shown that fumarate (and succinate) inhibit the activity or function of other members of the 2OG oxygenase superfamily, including histone demethylase enzymes (HDMs) and TET proteins which are critical in epigenetic regulation of gene expression (Xiao et al., 2012). Despite the identification of cancer-associated mutations in both classes of these enzymes, a direct causal role in oncogenesis is yet to be determined (Abdel-Wahab et al., 2009; van Haast et al., 2009; Dalgliesh et al., 2010; Ko et al., 2010).

SUCCINATION

In addition to its role as an allosteric regulator of 2OG-dependent oxygenases, fumarate is also an endogenous electrophile and reacts spontaneously with cysteine residues in proteins by a Michael addition reaction to form S-(2-succinyl) cysteine (2SC), a process termed succination (Alderson et al., 2006). Accumulation of cellular fumarate has been shown to correlate directly with an increase in succinated proteins. It has been proposed that this results from mitochondrial stress in adipocytes during adipogenesis, when cultured in high glucose medium, in adipose tissue of obese type 2 diabetic mice and in skeletal muscle of streptozotocin-induced type 1 diabetic rats (Fruzzell et al., 2011). Mechanistically, it has been proposed that nutrient excess from hyperglycemia results in high a NADH/NAD⁺ ratio, leading to feedback inhibition of oxidative phosphorylation and accumulation of mitochondrial intermediates including fumarate, which in turn causes protein succination (Fruzzell et al., 2012). Targets for succination include the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase, adiponectin, cytoskeletal proteins, and endoplasmic reticulum chaperone proteins. Furthermore, evidence suggests that succination of these proteins in cells may impair their functions (Blamak et al., 2008; Fruzzell et al., 2009).
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prevalent in many cancer types (Hayes and McMahon, 2009) and pathway is a clear point of focus for future work; especially as signaling or metabolic pathways.

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mice (DeNicola et al., 2011). NRF2 may contribute to tumor devel-

oncogene-induced Nrf2 transcription promotes tumorigenesis in the absence of electrophiles, through the activation of target genes con-

from the observation that there is striking upregulation of the nuclear factor (erythroid-derived 2)-like 2 (NRF2)-mediated antioxidant signaling pathway in our murine Fh1 deficient renal cyst model, mouse embryonic fibroblasts as well as human FH-deficient cells and tissues (Adam et al., 2011; Ooi et al., 2011).

NRF2 controls the adaptive response of cells to oxidative and electrophilic stress, through the activation of target genes contain-
ing antioxidant response elements (AREs) while KEAP1 is the substrate recognition subunit of a Cul3-based E3 ubiquitin ligase complex and a major cellular electrophile sensor (Zhang, 2010). In the absence of electrophiles, the homodimeric KEAP1 interacts with an NRF2 monomer, promoting its ubiquitylation and proteasomal-mediated degradation (McMahon et al., 2010).

KEAP1 has been shown to be succinated on two critical cysteine residues (Cys135 and Cys288) in FH-deficient cells, which disrupts

its interaction with NRF2, resulting in stabilization and accumula-
tion of nuclear NRF2 (Adam et al., 2011; Ooi et al., 2011). This allows binding to AREs and consequent activation of downstream target genes involved in defense against reactive oxygen species (Zhang, 2010). The activation of the NRF2-mediated antioxidant pathway is a clear point of focus for future work; especially as activating NRF2 mutations and inactivating KEAP1 mutations are prevalent in many cancer types (Hayes and McMahon, 2009) and oncogene-induced Nrf2 transcription promotes tumorigenesis in mice (DeNicola et al., 2011). NRF2 may contribute to tumor develop-
ment by enabling FH-deficient cells to tolerate high levels of exogenous or endogenous oxidants, thus promoting their survival.

Succination may result in the disrupted function of multi-
ple proteins and offers a unique mechanism by which fumarate may lead to dysregulated cellular metabolism and act as an oncometabolite. Clearly screens need to be undertaken to identify other candidate succination targets which have cysteine residues critical for their function and are associated with oncogenic signaling or metabolic pathways.

DISRUPTION TO METABOLISM

The Krebs cycle dysfunction caused by loss of FH activity poses

significant challenges to cells in meeting energy requirements, in the generation of macromolecular precursors and in sur-
vival. Studies, in part contradictory, using a number of cellular models, have identified a variety of mechanisms by which FH-deficient cells may deal with these problems. Impaired respiration and upregulation of aerobic glycolysis have been observed in FH-deficient cell lines and tissues, presumably as an adaptation to meet cellular energy requirements by producing ATP indepen-
dently of the TCA cycle (Sudarshan et al., 2009; O’Flaherty et al., 2010). Elevated glutaminolysis has been observed and stable iso-
tope labeling studies of an Fh1-deficient murine renal cell line have suggested that glutamine is the major carbon source for the Krebs cycle (Frezza et al., 2011b). These authors have also pro-
posed upregulation of the home biosynthesis pathway as a means of removing excess carbon from the dysregulated Krebs cycle whilst permitting partial mitochondrial NADH generation (Frezza et al., 2011b). Enhanced glycolysis and glutaminolysis are both stereotypic features of transformed cells (DeBerardinis et al., 2007; Vander Heiden et al., 2009) and may prime FH-deficient cells toward malignancy. Separately, partial reversal of the Krebs cycle, so called glutamine-dependent reductive carboxylation, has been observed in human carcinoma lines including UOK262 cells, defi-
cient in FH. By this mechanism 2OG is reductively carboxylated by IDH isoforms to generate isocitrate, followed by its subsequent metabolism to produce citrate, oxaloacetate and acetyl coenzyme A (AcCoA). AcCoA is crucial for fatty acid synthesis and protein acetylation while oxaloacetate is reduced to malate and eventually is decarboxylated to pyruvate. 

Antioxidant signaling pathway in our murine Fh1 deficient renal cell line has been observed and stable isotope labeling studies of an Fh1-deficient murine renal cell line have suggested that glutamine is the major carbon source for the Krebs cycle (Frezza et al., 2011b). These authors have also proposed upregulation of the home biosynthesis pathway as a means of removing excess carbon from the dysregulated Krebs cycle whilst permitting partial mitochondrial NADH generation (Frezza et al., 2011b). Enhanced glycolysis and glutaminolysis are both stereotypic features of transformed cells (DeBerardinis et al., 2007; Vander Heiden et al., 2009) and may prime FH-deficient cells toward malignancy. Separately, partial reversal of the Krebs cycle, so called glutamine-dependent reductive carboxylation, has been observed in human carcinoma lines including UOK262 cells, deficient in FH. By this mechanism 2OG is reductively carboxylated by IDH isoforms to generate isocitrate, followed by its subsequent metabolism to produce citrate, oxaloacetate and acetyl coenzyme A (AcCoA). AcCoA is crucial for fatty acid synthesis and protein acetylation while oxaloacetate is reduced to malate and eventually is decarboxylated to pyruvate.

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able to determine the endogenous levels of fumarate in different cells and under different stress conditions. Technical difficulties to undertaking this include heterogeneity in tissue samples and, more significantly, the lack of effective methods to accurately quantify small molecule metabolites such as succinate and fumarate in sub-cellular compartments, e.g., mitochondria and nucleus, where local metabolism is tightly regulated.

The shift in focus of cancer research to one of trying to understand how altered cellular metabolism and cancer is linked has highlighted how woefully ignorant we are about the complexities and interrelationships of cellular metabolic pathways and how these are altered under conditions of a variety of stress agents. However, studies into rare genetic disorders associated with metabolism are beginning to provide real insights into the adaptative responses of cells and dysregulated metabolism associated with cancer.

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