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The reduced concentration of citrate in cancer cells: An indicator of cancer aggressiveness and a possible therapeutic target

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ABSTRACT

Proliferating cells reduce their oxidative metabolism and rely more on glycolysis, even in the presence of O_2 (Warburg effect). This shift in metabolism reduces citrate biosynthesis and diminishes intracellular acidity, both of which promote glycolysis sustaining tumor growth. Because citrate is the donor of acetyl-CoA, its reduced production favors a deacetylation state of proteins favoring resistance to apoptosis and epigenetic changes, both processes contributing to tumor aggressiveness. Citrate levels could be monitored as an indicator of cancer aggressiveness (as already shown in human prostate cancer) and/or could serve as a biomarker for response to therapy. Strategies aiming to increase cytosolic citrate should be developed and tested in humans, knowing that experimental studies have shown that administration of citrate and/or inhibition of ACLY arrest tumor growth, inhibit the expression of the key anti-apoptotic factor Mcl-1, reverse cell dedifferentiation and increase sensibility to cisplatin.

1. Introduction

It is well established that cellular respiration predominantly occurs under aerobic conditions (aerobic respiration) in eukaryotic cells, though under certain conditions eukaryotic cells can also produce energy anaerobically. These two processes share a common initial process, called glycolysis, notably regulated by phosphofructokinase 1 (PFK 1), resulting in the production of pyruvate from glucose. Under aerobic conditions, pyruvate enters the tricarboxylic acid (TCA) cycle to produce citrate and high levels of ATP, whereas under anaerobic conditions, pyruvate produces lactate and very low amounts of ATP. In 1857, Pasteur demonstrated that yeast cells typically cultivated under hypoxic conditions displayed a reduced rate of glycolysis and a lower production of lactate when they were exposed to oxygen. This "Pasteur effect" is due to an increase in the mitochondrial production of ATP and citrate, which exerts a negative feedback on PFK 1, hence inhibiting glycolysis (Lehninger, 1975). Therefore, these molecules constitute a sort of "fuel gauge" for cells: when elevated, gluconeogenesis and glycogen synthesis are activated, and acetyl-CoA (derived from citrate) is used in fatty acid biosynthesis. By contrast, when their levels are low, glycolysis is activated (Icard and Lincet, 2012).

Cancer cells, especially rapidly growing tumor cells, such as ascites, are able to reduce oxidative phosphorylation (OXPHOS), to enhance the rate of glycolysis and lactate production (fermentation), even under aerobic conditions, a process known as the "Warburg effect" (Warburg, 1930; Warburg, 1956). Since this process is common to many cancer cells, we hypothesized that the regulation of the "Pasteur effect" was somehow blocked or inoperative in these cells. By adding exogenous citrate to the culture medium of cancer cells, we previously observed an arrest in cell growth, an increase in cancer cell apoptosis as well as in their sensitivity to cisplatin (Zhang et al., 2009). In the current review, we initially summarize the results of *in vitro* experiments, demonstrating an anti-cancer effect linked to the administration of citrate (Zhang et al., 2009; El Sayed et al., 2012a,b; Hanai et al., 2012; Kruspig et al., 2012; Lincet et al., 2013; Lu et al., 2011), before presenting evidence that the "Warburg effect" promotes aggressiveness and growth of tumor cells by decreasing the mitochondrial production of citrate, ATP and CO_2. Consequently, we also link this reduction in citrate production to intracellular alkalinization

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and protein deacetylation, both providing favorable conditions for tumor development. Finally, we conclude that quantification of citrate concentration in cancer tissues might serve as a metabolic biomarker of disease prognosis, as recently demonstrated for prostate cancer (Giskeodegard et al., 2013), whereas therapeutic strategies designed to increase citrate levels may be considered for rendering cells more sensitive to chemotherapy.

1.1. Citrate administration has an anti-proliferative effect: experimental evidence

Several *in vitro* studies (Zhang et al., 2009; Lincet et al., 2013; Lu et al., 2011) have shown that the exogenous administration of citrate inhibits the growth of various cancer carcinoma cell lines (mesothelioma, stomach, ovarian) in a dose-dependent manner. Indeed, clear features of apoptosis (activation of PARP and caspase 3) and inhibition of the expression of the anti-apoptotic factor Mcl-1 were observed (Lincet et al., 2013; Lu et al., 2011; Kafara et al., 2015). In a mesothelioma cell line highly resistant to cisplatin, and in which 10 mM citrate were required to arrest cell growth, the combination of cisplatin and citrate was lethal (Zhang et al., 2009). Several authors have also confirmed the ability of citrate to promote apoptosis in other cancer cell lines including neuroblastoma, glioma, non-small lung cancer, and more (El Sayed et al., 2012a,b; Hanai et al., 2012; Kruspig et al., 2012). Furthermore, in a non-small cell lung cancer cells, citrate supplementation (above 5 mM) induced additional apoptosis in ATP-citrate lyase (ACLY) knockdown cells (Hanai et al., 2012).

The deleterious effects of citrate for cancer cells remain to be studied: they were found to be related to the activation of the extrinsic apoptotic pathway (expression of caspases-8 and −2), and to decreased of ATP production (Kruspig et al., 2012).

1.2. The decrease in citrate production by the TCA is linked to tumor aggressiveness

Two processes combine to decrease the mitochondrial production of ATP and citrate in aggressive cancer cells, namely the loss of mitochondria and the reduced activities of the TCA cycle and OXPHOS. Otto Warburg thought that aerobic fermentation was due to the impairment of mitochondrial respiration, and considered that this impairment was the "signature" of cancer cells (Warburg, 1956; Koppenol et al., 2011), strengthened in his conviction by the few mitochondria observed in these cells. This observation was confirmed in later studies reported by Pedersen (1978), and others (Rossignol et al., 2004, 2000), who concluded that poorly differentiated fast growing cancer cells had fewer mitochondria. Notably, a recent study conducted on 346 human breast cancer samples revealed the presence of mitochondria in low-grade cancers, while they were scarce or absent in most aggressive tumors, according to electron micrographs (Elliott and Barnett, 2011).

Numerous observations also described morphological anomalies of mitochondria (Pedersen, 1978; Boland et al., 2013; Mates et al., 2009; Benard and Rossignol, 2008), as well as functional abnormalities of their respiratory chain (Brandon et al., 2006; Chatterjee et al., 2006; Grandemange et al., 2009; King et al., 2006; Lopez-Rios et al., 2007; Matoba et al., 2006). Presumably, some anomalies, in particular the deficiency in the ATPase complex (Cuezva et al., 2002) and/or the overexpression of the uncoupling protein UCP2 (Derdak et al., 2008; Samudio et al., 2009), reduced the production of ATP by OXPHOS. However, it is not known whether or not these anomalies are present in a majority of cancer cells. Furthermore, the average ATP produced during OXPHOS was similar in numerous cancer cells when compared to normal cells (Zu and Guppy, 2004). It is noteworthy that the progression of tumor growth is critically dependent on oxygen and nutrient supply and the drainage of metabolites by heterogeneous micro vessels (Folkman, 1995). Oxygen concentration rapidly decreases within tumors since diffusion without the involvement of blood vessels allows transport processes only over very short distances. (Folkman, 1995; Vaupel et al., 2003; Multhoff et al., 2014). These hostile conditions force tumor cells to enhance the "Warburg effect" and select "robust" cells, which develop other several pro-survival features and traits of aggressiveness, such as the expression of anti-apoptotic factors promoting an anticancer drug resistance phenotype (Bindra et al., 2007; Pouyssegur et al., 2006; Xu et al., 2005; Yuan et al., 2000; Brotin et al., 2010; Zhitomirsky and Assaraf, 2016; Gonen and Assaraf, 2012; Raz et al., 2014; Gonen and Assaraf, 2010). Importantly, Pedersen reported that rapidly growing (or poorly differentiated) cancer cells reduced their OXPHOS-ATP production, while they had more glycolytic enzyme activity (Pedersen, 1978). He showed that mitochondria account for 60–85% of the ATP produced in these cells, a much lower yield compared to the 80–95% level found in cancer cells which proliferate at a slow or intermediate rate. Finally, this study revealed that in rapidly proliferating cancer cells, such as ascites, the rate of mitochondrial ATP production drops to a mere 50% (Pedersen, 1978). Interestingly, a recent study conducted on human colon cancer cells revealed that a decrease in the mitochondrial ATP production was correlated with tumor aggressiveness in the context of resistance to chemotherapy (Zhou et al., 2012). Given the tight relationship between ATP and citrate production, one would speculate that citrate production may concomitantly be reduced in cancer cells or be associated with tumor aggressiveness, as recently demonstrated in the context of human prostate cancer (Giskeodegard et al., 2013).

In order to proliferate more rapidly, cancer cells must drastically increase their glucose consumption, to supply essential metabolic nutrients including ribose, glycerol and serine, necessary for various biosynthetic pathways (Icard and Lincet, 2012; Vander Heiden et al., 2009). Under normoxic conditions, proliferating cancer cells must reduce their mitochondrial production of ATP and citrate to prevent the accumulation of these molecules following the consumption of large amounts of glucose, which would lead to the inhibition of glycolysis. Several processes are involved to allow cancer cells to metabolize the large amounts of glucose, without provoking the deleterious overproduction of citrate: (i) an increase in glutaminolysis (oxidation of glutamine). This pathway reduces the production of citrate because it sustains the biosynthesis of various macromolecules (Mates et al., 2009; Deberardinis and Cheng, 2010; Dang, 2013), whereas it produces fewer ATP molecules than the oxidation of one pyruvate derived from glucose (9 ATP *vs* 15 ATP); (ii) the induction of hypoxia-inducible factor (HIF-1), which upregulates, even under normoxic conditions, several glycolytic enzymes (Marin-Hernandez et al., 2009) and activates pyruvate dehydrogenase kinase 1 (PDK 1), leading to the inactivation of pyruvate dehydrogenase (PDH) (Kim et al., 2006; Papandreou et al., 2011). This inhibition promotes a disassociation between pyruvate and the TCA cycle, leading to reduced production of citrate; (iii) the overexpression of the key anti-apoptotic Bcl-x_L molecule. This latter factor inhibits the citrate carrier (CIC) (Alavian et al., 2011; Catalina-Rodriguez et al., 2012), hence reducing the mitochondrial export of citrate; (iv) the "Crabtree effect", also participates in reducing the production of citrate in cancer cells (Redman et al., 2013; Suchorolski et al., 2013). This effect is simply defined as the observation that cancer cells cultured with high concentration of glucose, decrease their mitochondrial respiration (Crabtree, 1929). In yeast, the "Crabtree effect" is mediated by 1,6-biphosphate (F1,6-BP) which inhibits mitochondrial OXPHOS activity at complex IV and thereby down-regulates the TCA cycle (Diaz-Ruiz et al., 2008). However, the reduced level of citrate in cancer results not only from a lower production of citrate by the mitochondria, but also from the cleavage of citrate by ACLY, an enzyme often expressed in