The Shifting Balance Between CLU Forms During Tumor Progression

Sabina Pucci* **and Saverio Bettuzzi**†

**Department of Biopathology, University of Rome "Tor Vergata," Rome, Italy*
†Dipartimento di Medicina Sperimentale, Sezione di Biochimica, Biochimica Clinica e Biochimica dell'Esercizio Fisico, Via Volturno 39-43100 Parma and Istituto Nazionale Biostrutture e Biosistemi (I.N.B.B.), Rome, Italy

Cell transformation is strictly linked to important metabolic changes which are instrumental for initial survival of cancer cells and subsequent spreading of disease. Early (i.e., anerobic glycolysis) and late metabolic changes (i.e., fatty acid metabolism) are required for progression and clinical emergence of cancer. Besides well-known tumor suppressors and oncogenes, several metabolic genes have been found implicated in this multistep process, among which are fatty acid synthase (FASN) and carnitine palmitoyl transferase I (CPT I). An intriguing link between these metabolic shifts and a change in the balance between nuclear and secreted forms of CLU (nCLU/sCLU) has been suggested. The shifting balance between CLU forms during tumor progression, by affecting the fate of the cell, seems to be strongly influenced by the metabolic shift occurring in the different steps of tumor progression.

I. INTRODUCTION

In tumor progression, the overall metabolic demand of neoplastic cells is significantly higher than most other tissues and the cancer cell depends more on glycolysis, even in the presence of available oxygen. Enhanced "aerobic glycolysis" in cancer cells is known as the Warburg effect (Shaw, 2006). Though controversial over the years, a molecular basis for the Warburg effect is emerging from genetic and pharmacological studies which demonstrate that specific oncogene and tumor suppressor mutations or dysregulation directly control glycolysis and oxidative phosphorylation. The combination of these mutations and the hypoxic conditions in many tumor types is likely to synergize and to control the CLU expression depending on the overall

Advances in CANCER RESEARCH

0065-230X/09 $35.00
DOI: 10.1016/S0065-230X(09)04003-2

metabolic state of individual tumors. Therefore, changes in CLU forms expression could be strongly influenced by the metabolic shift occurring in the different steps of tumor progression.

II. SHIFTING OF CELL METABOLISM IN TUMORIGENESIS

Mutated cancer cells could benefit from glycolysis in many ways, in fact glycolysis generates more energy more quickly than in normal cells: unlike to the normal cell, the neoplastic cell typically utilizes nutrients in an "energy-independent" manner, as a consequence it does not need to break down amino acids and fatty acids to generate energy and it can utilize them to build proteins and lipids necessary for growth. The proliferative advantage of glycolysis is further demonstrated by *in vitro* experiments showing that the switch back to oxidative phosphorylation from glycolysis is accompanied by a decrease in cell growth and tumorigenicity. The long-chain fatty acids accumulated can be toxic and also induce cell death. Hence, we can speculate that the shifting of cell metabolism controls tumor cell growth and apoptosis. The nutrient deprivation and energy stress in nonmutated cell activate a program that inhibits cell-cycle progression and biosynthetic process through LKB1-activated AMPK. On the other hand, when cell lacks tumor suppressors (such as TSC2, LKB1, p53) or carries active oncogenes (Ras, Akt, Her2) mTOR and HIF1 pathways are activated, increasing cell growth and protein and lipid synthesis through fatty acid synthase (FASN) overexpression.

FASN catalyzes the synthesis of palmitate from the condensation of malonyl-CoA and acetyl-CoA and plays an important role in energy homeostasis by converting excess carbon intake into fatty acids for storage. In normal cells, FASN is expressed at low levels due to the presence of dietary lipids. In contrast, neoplastic cells can either use endogenously synthetic fatty acids to satisfy their metabolic necessities and to support membrane synthesis. Menendez *et al.* (2005a,b) show that the extracellular acidosis present in the microenvironment of solid tumors can work in an epigenetic fashion by upregulating the transcriptional expression of FASN gene in breast cancer cells. Moreover, The PI3K/AKT signaling pathway has also been implicated in the regulation of FASN expression. A positive feedback loop has been proposed between AKT activation and FASN expression (Wang *et al.*, 2005). In fact, the well-characterized oncogene Her-2/Neu and its downstream effector PI3K have a stimulatory effect on FASN gene. It has been shown that FASN expression is markedly increased in several human malignancies, notably breast and prostate cancer, and its

overexpression in tumor tissues from patients with colon, breast and prostate carcinomas as well as melanoma and gastrointestinal stromal tumors has been associated with a poor prognosis (Pizer *et al.*, 2001). In addition, one-fourth of human prostate cancers have genomic amplification of FASN (Rossi *et al.*, 2003).

It has been recently demonstrated that FASN is overexpressed also in prostate intraepithelial neoplasia (PIN) compared with adjacent normal tissue, suggesting that it plays a role in the initial phases of prostate tumorigenesis, and in metastatic prostate cancer, suggesting that it may function as a mediator of biological aggressiveness. Importantly, Rossi (Rossi *et al.*, 2003) demonstrated that FASN-overexpressing prostate cancers display a characteristic gene expression signature, indicating a particular transcription pattern related to its activity. Its role in cancer supports the hypothesis that FASN is a metabolic enzyme and candidate oncogene in cancer (Migita *et al.*, 2009).

Overall these data support the role of FASN as a novel "*metabolic oncogene*" in cancer cells (Menendez *et al.*, 2005a,b). From a functional standpoint, pharmacological approaches to decrease expression of FASN have been shown to result in growth inhibition of various tumor cell lines, including those derived from prostate cancer and/or prostate cancer tumor xenografts *in vivo* (Migita *et al.*, 2009). In addition, functional interference, mostly by RNA interference, has been shown to result in G1 arrest and/or induction of apoptosis. FASN-specific inhibitors such as mycotoxin cerulenin and its derivative C75, the beta-lactone orlistat, the green tea polyphenol EGCG, and the novel and potent inhibitors of FASN derived from green tea catechins (GTC) have been reported to induce programmed cell death in cancer cells (Zhang *et al.*, 2008).

III. SHIFTING OF CLU FORMS DURING TUMOR PROGRESSION

Pucci S. and her group have found that the increasing endogenously synthesized fatty acids together with increasing levels of IL-6, also induced by Her2/Neu signaling and sustained in breast cancer by autocrine loop, induce high level of prosurvival sCLU in human breast carcinomas and *in vitro* breast cancer cell lines with or without Her2 gene amplification (SKBR3, MCF7 cells). The expression levels of FASN would also be in breast cancer cells, an indicator of Her2 transduction activity. The amplification of Her2 is present in 25% of breast cancer but it could be commonly present in various epithelial tumors that overexpress FASN. FASN-derived phospholipids, the end product of nearly 85% of all lipids synthesized *de novo* by

FASN in tumor cells, have been observed to end up in lipid rafts in plasma membranes (Menendez *et al.*, 2005a,b). In tumor progression, general alterations in the lipid compositions of the cellular and mitochondrial membranes may confer a selective growth advantage to aberrant cells that display increased FASN activity by inhibition of apoptosis. A strong increase of sCLU in the cytoplasm of tumoral tissues was correlated with FASN protein levels. sCLU, which binds hydrophobic macromolecules, would act in this context to "clear" potentially harmful cellular components, enhancing survival of cancerous cell.

Treatment *in vitro* with hydrocortisone strikingly induces an increase of sCLU protein. A strong increase of sCLU was found in FASN overexpressing breast tumors, which was detected in the cytoplasm bound to Ku70-Bax complex, inhibiting Bax-dependent cell death activation in breast cancer cells. These interactions among Ku–CLU–Bax represent one of cell death escaping mechanism common in colon cancers as previously published by Pucci *et al.* (2009). FASN inhibition by cerulenin induces an increased expression and the accumulation of nCLU in the nuclei of breast cancer cells, more evident in the Her2-amplified SKBR3 cells, favoring the proapoptotic pathway reverting the neoplastic apoptosis resistance.

Moreover, FASN inhibitors induced an increase of Ku70 acetylation and subsequent sCLU–KU70 release from Bax, sterically inhibited in tumors by these interactions. Therefore, the apoptotic processes induced through the inhibition of the oncogene FASN involve BAX heterodimerization and migration to mitochondria and the accumulation of nCLU in the nuclei. These observations suggest that FASN overexpression may protect prostate epithelial cells from apoptosis, while inhibition of FASN expression could induce the proapoptotic form of CLU in cancer cells.

All together, data suggest a link among tumor progression, cell metabolic shift, sCLU/nCLU balance, and cell fate in neoplastic cells. These observations on breast and colon cancer cells are in agreement with data obtained by Bettuzzi and his group in TRAMP mouse prostate carcinoma model using GTC, known to inhibit the activity of FASN (Scaltriti *et al.*, 2006).

They reported the effect of the GTC, known to display chemopreventive effects in many cancer models, including transgenic adenocarcinoma mouse prostate (TRAMP) mice that spontaneously develop prostate cancer (CaP) on nCLU production. In particular, CLU expression was detectable at basal levels in young TRAMP mice, being potently downregulated together with caspase-9 during onset and progression of CaP. At difference, in TRAMP mice treated with the FASN inhibitors GTC, tumor progression was chemoprevented and CLU mRNA and protein progressively accumulated in the prostate gland, while caspase-9 expression also returned to basal levels. Massive nuclear staining with anti-CLU antibodies of prostate cells (nCLU) was demonstrated at early stages of chemoprevention treatment

with GTC. These data suggested CLU as tumor suppressor in prostate cancer, possibly mediating the anticancer effect of GTC (Caporali *et al.* 2004). Interestingly, in a further study (Scaltriti *et al.*, 2006), molecular classification of GTC-sensitive versus GTC-resistant prostate cancer was successfully attempted in the TRAMP mice model by quantitative real-time PCR gene profiling. A set of eight informative genes previously identified (Bettuzzi *et al.*, 2003) was used for molecular classification. Linear discriminant analysis was performed to discriminate four mice classes: wild type, TRAMP spontaneously developing CaP, GTC-sensitive (chemoprevented) TRAMP, and GTC-resistant TRAMP in which administration of GTC failed to prevent CaP progression. In this study, different combinations of two genes at a time extracted from the whole set of eight genes (with a total of 28 different possible combinations) were taken into consideration. Among these combinations, best performing one was CLU-GAPDH, a well-known enzyme of the glycolysis pathway, with a 0% misclassification ratio over the four classes studied. As described previously, this result connecting dysregulation of CLU expression and glycolysis in cancer cells can be interpreted as due to the metabolic shift occurring in the different steps of tumor progression as hypothesized by the Warburg effect.

Besides the role of FASN in tumor development and progression, it is to note the behavior of another "player" of cell metabolism, carnitine palmitoyl transferase I (CPT I) and its new role in the regulation of DNA acetylation and CLU expression in the tumoral context. CPT I in normal cells resides at the outer mitochondrial membrane and it serves to transport long-chain fatty acids into mitochondria for beta-oxidation. Physiologically, the overexpression of FASN downmodulates CPT I activity, reducing oxidation of newly synthesized fatty acids suggesting a reciprocal regulation that could become aberrant in neoplastic cells.

Two isoforms of CPT1 have been characterized, known as L-CPT1 (CPT1A) and M-CPT1 (CPT1B) in liver (L-) and muscle (M-), respectively, where the expression of each was initially described, showing overlapping tissue-specific expression. While CPT1B is expressed in skeletal muscle, heart, testis, and adipose tissue, CPT1A has a more widespread distribution (Weis et al, 1994). CPT I isoform switching has been shown to take place in development when programmed cell death is enhanced.

The precursors of sphingolipids like palmitoyl-CoA are subject to removal from the cytoplasm by CPT I, thus CPT I activity may limit *de novo* synthesis of sphingolipids. Therefore, a feedback loop transregulate the activities of FASN and CPTI. The long-chain fatty acids such as palmitate and stearate can cause programmed cell death correlated with *de novo* synthesis of ceramide (Paumen *et al.*, 1997). It was demonstrated that CPT I interacts with Bcl-2 protein, that regulates programmed cell death in several systems and it is also expressed at the outer mitochondrial membrane (Reed, 1994).

Bcl-2 binding to CPT I may modulate sphingolipid metabolism in a yet to be defined way and it would control a cell death-specific activity of CPT I at the mitochondrial membrane. In addition, the carnitine system (which comprises carnitine, CPT I, carnitine acetyl transferase, and carnitine translocase) plays an important role in the cell-trafficking of short-chain fatty acids such as acetyl-CoA and works to maintain the acetyl-CoA/CoA ratio (Bremer, 1997).

Therefore, CPT I activity would confer a protective effect on normal cell viability, by the clearance of long-chain fatty acyl-CoA from the cytoplasm.

Mazzarelli *et al.* (2007) recently observed that the CPT I was significantly decreased in the mitochondria, and it strikingly localized in the nuclei of tumoral tissues (colon, breast, liver, ovary). At this purpose, *in vitro* experiments using epithelial neoplastic (MCF-7, Caco-2, HepG2) cells and nonneoplastic cell lines (MCF-12F) confirmed a nuclear localization of CPT1 protein exclusively in neoplastic cells. Moreover, histone deacetylase (HDAC) activity showed significantly higher levels in nuclear extracts from neoplastic than from control cells. HDAC1 and CPT1 proteins were coimmunoprecipitated in nuclear extracts from MCF-7 cells. The treatment with HDAC inhibitors such as trichostatin A and butyrate significantly decreased nuclear expression of CPT1 and its binding to HDAC1. The existence of CPT1A mRNA transcript variant 2 in MCF-7, besides the classic isoform 1 was also characterized. Mazzarelli *et al.* observed that CPT I in the nucleus could be implicated in the epigenetic regulation of gene transcription, a relevant process to control tumor growth. In fact, the peculiar localization of CPT1 in the nuclei of human carcinomas and the disclosed functional link between nuclear CPT1 and HDAC1 propose a new role of CPT1 in the histonic acetylation level of tumors. In neoplastic cells FASN overexpression inhibited β-oxidation, thus CPT I protein possibly could move to the nucleus and modulate the acetyl moieties at histone level. Moreover, the silencing of CPT1A nuclear expression by small-interfering RNAs is a sufficient condition to induce apoptosis in MCF-7 breast cancer cells where FASN and sCLU are concomitantly overexpressed. The apoptosis triggered by RNA interference correlates with reduction of HDAC activity and hyperacetylation of histone- and nonhistone proteins, involved in cancer-relevant death pathways. Moreover, the CPT1A knockdown induces downstream effects on proapoptotic genes (upregulation) and invasion- and metastasis-related genes (downmodulation), as shown by microarray analysis. Downstream effects induced by histone hyperacetylation were the upregulation of proapoptotic transcription (BAD, CASP9, COL18A1) and the downmodulation of invasion and metastasis-related genes (TIMP-1, PDGF-A, SERPINB2). Focusing on the cell-death pathway, CPT1A silencing induced cell death modulating Ku70 acetylation state and affecting sCLU–Ku70–Bax interactions and inducing nCLU accumulation in the nucleus.

IV. CONCLUDING REMARKS

The results provided above bring important evidences of existing mechanisms linking tumor progression to the metabolism-dependent epigenetic control to cell-death escape. In particular, FASN and CLU form production are specifically regulated in cancer cells which merely retain CPT1A nuclear localization. The possibility to revert these aberrant interactions by RNA targeting could offer new strategies for innovative cancer therapies.

ACKNOWLEDGMENTS

S.B. grant sponsors:

– FIL 2008 and FIL 2009, University of Parma, Italy; AICR (UK) Grant No. 06-711; Istituto Nazionale Biostrutture e Biosistemi (INBB), Roma, Italy.

– Alleanza Contro ilCancro (ACC)-Istituto superiore di Sanità (ISS) Art.3 DM 21 luglio 2006-Programma Straordinario di Ricerca Oncologica 2006-Programma 3; Progetto ordinario IRCCS.

REFERENCES

Bettuzzi, S., Scaltriti, M., Caporali, A., Brausi, M., D'Arca, D., Astancolle, S., Davalli, P., and Corti, A. (2003). Successful prediction of prostate cancer recurrence by gene profiling in combination with clinical data: A 5 years follow-up study. *Cancer Res.* **63,** 3469–3472.

Bremer, J. (1997). The role of carnitine in cell metabolism. *In* "Carnitine Today," (C. De Simone and G. Famularo, Eds.), pp. 1–37. Springer-Verlag, Heidelberg.

Caporali, A., Davalli, P., Astancolle, S., D'Arca, D., Brausi, M., Bettuzzi, S., and Corti, A. (2004). The chemopreventive action of catechins in the TRAMP mouse model of prostate carcinogenesis is accompanied by clusterin over-expression. *Carcinogenesis* **25,** 2217–2224.

Mazzarelli, P., Pucci, S., Bonanno, E., Sesti, F., Calvani, M., and Spagnoli, L. G. (2007). Carnitine palmitoyltransferase I in human carcinomas: A novel role in histone deacetylation? *Cancer Biol. Ther.* **6,** 1606–1613.

Menendez, J. A., Vellon, L., and Lupu, R. (2005a). Targeting fatty acid synthase-driven lipid rafts: A novel strategy to overcome trastuzumab resistance in breast cancer cells. *Med. Hypotheses* **64,** 997–1001.

Menendez, J. A., Decker, J. P., and Lupu, R. (2005b). In support of fatty acid synthase (FAS) as a metabolic oncogene: Extracellular acidosis acts in an epigenetic fashion activating FAS gene expression in cancer cells. *J. Cell. Biochem.* **94**(1), 1–4.

Migita, T., Ruiz, S., Fornari, A., Fiorentino, M., Priolo, C., Zadra, G., Inazuka, F., Grisanzio, C., Palescandolo, E., Shin, E., Fiore, C., Xie, W., *et al.* (2009). Fatty acid synthase: A metabolic enzyme and candidate oncogene in prostate cancer. *J. Natl Cancer Inst.* **101**(7), 519–532.

Paumen, M. B., Ishida, Y., Muramatsu, M., Yamamoto, M., and Honjo, T. (1997). Inhibition of carnitine palmitoyltransferase I augments sphingolipid synthesis and palmitate-induced apoptosis. *J. Biol. Chem.* **272**(6), 3324–3329.

Pizer, E. S., Pflug, B. R., Bova, G. S., Han, W. F., Udan, M. S., and Nelson, J. B. (2001). Increased fatty acid synthase as a therapeutic target in androgen-independent prostate cancer progression. *Prostate* **47**(2), 102–110.

Pucci, S., Mazzarelli, P., Sesti, F., Boothmann, D. A., and Spagnoli, L. G. (2009). Interleukin-6 affects cell death escaping mechanisms acting on Bax–Ku70–Clusterin interactions in human colon cancer progression. *Cell Cycle* **8**(3), 473–481.

Reed, J. C. (1994). Bcl-2 and the regulation of programmed cell death. *J. Cell Biol.* **124**(1–2), 1–6.

Rossi, S., Graner, E., Febbo, P., Weinstein, L., Bhattacharya, N., Onody, T., Bubley, G., Balk, S., and Loda, M. (2003). Fatty acid synthase expression defines distinct molecular signatures in prostate cancer. *Mol. Cancer Res.* **1**(10), 707–715.

Scaltriti, M., Belloni, L., Caporali, A., Davalli, P., Remondini, D., Rizzi, F., Astancolle, S., Corti, A., and Bettuzzi, S. (2006). Molecular classification of green tea catechins-sensitive and -resistant prostate cancer in the TRAMP mice modelby quantitative real-time PCR gene profiling. *Carcinogenesis* **27**, 1047–1053.

Shaw, R. J. (2006). Glucose metabolism and cancer. *Curr. Opin. Cell Biol.* **18**, 598–608.

Wang, H. Q., Altomare, D. A., Skele, K. L., Poulikakos, P. I., Kuhajda, F. P., Di Cristofano, A., and Testa, J. R. (2005). Positive feedback regulation between AKT activation and fatty acid synthase expression in ovarian carcinoma cells. *Oncogene* **24**(22), 3574–3582.

Weis, B. C., Esser, V., Foster, D. W., and McGarry, J. D. (1994). Rat heart expresses two forms of mitochondrial carnitine palmitoyltransferase I. The minor component is identical to the liver enzyme. *J. Biol. Chem.* **269**, 18712–18715.

Zhang, S. Y., Ma, X. F., Zheng, C. G., Wang, Y., Cao, X. L., and Tian, W. X. (2008). Novel and potent inhibitors of fatty acid synthase derived from catechins and their inhibition on MCF-7 cell. *J. Enzyme Inhib. Med. Chem.* **1**, 1.