Proline metabolism and cancer

James M. Phang¹, Wei Liu¹

¹Metabolism and Cancer Susceptibility Section, Laboratory of Comparative Carcinogenesis, Center for Cancer Research, National Cancer Institute at Frederick, Frederick, MD 21702, USA

TABLE OF CONTENTS

1. Abstract
2. Introduction
   2.1. Proline cycle
   2.2. P5C reductase
   2.3. Proline oxidase
   2.4. Tumor microenvironment
3. POX as metabolic tumor suppressor
   3.1. Mechanisms characterized in tissue culture
   3.2. POX suppresses tumors in xenograft tumors
   3.3. POX is downregulated in human tumors
4. Mechanism for POX downregulation in tumors
   4.1. Identification of miR-23b*
   4.2. POX and miR-23b* in human clear cell renal carcinomas
   4.3. Reversal of POX suppression by inhibiting miR-23b*
5. Other mechanisms for inducing POX
   5.1. PPARgamma
   5.2. Physiologic ligand for PPARgamma upregulates POX
   5.3. PPARgamma and autophagy
6. Autophagy and survival
   6.1. POX and nutrient deprivation in autophagy
   6.2. Proline and glutamine
   6.3. Proline and hydroxyproline in anaplerosis
7. Metabolism in the tumor microenvironment
   7.1. Glucose and glutamine in tissue culture
   7.2. Degradation of extracellular matrix by MMPs
   7.3. MMPs and tumorigenesis
8. Metabolic timeline in tumorigenesis
9. Perspective
10. Acknowledgements
11. References

1. ABSTRACT

Proline plays a special role in cancer metabolism. Proline oxidase (POX), a.k.a. proline dehydrogenase (PRODH), is among a few genes induced rapidly and robustly by P53, the tumor suppressor. Ectopic expression of POX under control of tet-off promoter initiated mitochondrial apoptosis. The mechanism activated by POX is mediated by its production of ROS. In immunodeficient mice, POX overexpression markedly retarded growth of xenograft tumors. In human tumors of the digestive tract and kidney, POX was markedly decreased, suggesting that the suppressive effect of POX was downregulated. This was not due to POX gene mutations or hypermethylation. Instead, a microRNA, miR-23b*, expressed at high levels in tumors, was a potent inhibitor of POX expression. Furthermore, antagonism of miR-23b* reversed the downregulated expression of POX and its tumor-suppressive effect, thereby providing a therapeutic strategy. POX not only responds to genotoxic stress, but also to inflammatory and metabolic stress. Depending on microenvironmental and temporal factors, POX can mediate oppositely-directed responses—programmed cell death, on the one hand, and survival, on the other.

2. INTRODUCTION

2.1. Proline cycle

In his 1970 landmark review of proline and hydroxyproline metabolism, Elijah Adams emphasized the interconversions of proline, glutamate and ornithine (1). The discovery and description of familial hyperprolinemias by Scriver and Efron (2) led to the enzymatic characterization of the mechanism underlying this inborn error of metabolism (3). Intensive studies in tissue culture based on the revealed biochemistry together with the use of metabolic intermediates led us to propose that the proline metabolic system may play a regulatory role in intermediary metabolism (4). As the only proteinogenic secondary amino acid, proline has features which are advantageous for this regulatory function. The inclusion of its alpha-nitrogen within a pyrrolidine ring sequesters proline from the generic amino acid enzymes, the decarboxylases and transaminases which metabolize other amino acids. Instead, the metabolism of proline is restricted to its own family of enzymes with their cellular and organellar localization and their own regulatory
Proline metabolism and cancer

**Figure 1.** A schematic of the proline metabolic pathway and the “proline cycle.” Glutamic-gamma-semialdehyde (GSA) is in spontaneous equilibrium with its cyclized tautomer, delta-1-pyrroline-5-carboxylate (P5C); it is the obligate intermediate in the carbon interchange between the urea cycle and TCA cycle. The proline cycle transfers reducing equivalents to mitochondria; it is catalyzed by proline oxidase and P5C reductase. Proline oxidase is bound to mitochondrial inner membranes. Recent discoveries have identified 3 isozymes of P5C reductase (not shown) with distinct intracellular localization allowing for putative redox transfers within and between cellular compartments (9). The abbreviations shown are as follows: PRO, L-proline; P5C, delta-1-pyrroline-5-carboxylate; GLU, glutamate; GSA, glutamic-gamma-semialdehyde; ORN, ornithine; alpha-KG, alpha-ketoglutarate; TCA, tricarboxylic acid; NAD(P), nicotinamide adenine dinucleotide (phosphate). Enzymes are designated as follows: 1, P5C synthase; 2, P5C reductase (generic designation, there are 3 isozymes); 3, proline oxidase, a.k.a. proline dehydrogenase; 4, P5C dehydrogenase; 5, ornithine aminotransferase; 6, spontaneous.

mechanisms which mediate these special functions. These early findings were reviewed and the regulatory functions of “the proline cycle” was proposed in 1985 (4) (Figure 1).

2.2. P5C reductase

From the vantage point of the 3rd Triennial International Symposium on Proline Metabolism, it is gratifying to see the advances in the field over the years and especially during the last decade. In the late 1990s and early 2000s, considerable advances were made in understanding the function of proline oxidase a.k.a. proline dehydrogenase (5-7). Recent advances have focused on the synthetic half of the proline cycle. The discovery of pyrroline-5-carboxylate reductase mutations in humans (8) suggest a number of novel mechanisms resulting in the observed phenotype. Additionally, investigators have identified and characterized three isozymes of P5C reductase, each with its subcellular localization and pyridine nucleotide preference (9). These discoveries have arrived at a propitious time since we are in the midst of a sweeping resurgence of scientific interest in metabolism (10-12). It is gratifying that proline may be the basis for an important regulatory paradigm in intermediary metabolism.

Our laboratory focused on pyrroline-5-carboxylate reductase in the 1980s (4). We found that the human erythrocyte enzyme, purified and characterized, had kinetic properties and pyridine nucleotide preferences different than that from other cells and tissues (13). This allowed us to define the proline cycle (Figure 1) with the transfer of reducing potential from cytosol to mitochondria. However, the relevance of the proline cycle was questioned since its contribution to metabolism and regulation did not fit into the general physiologic paradigm. We previously speculated that the system functioned as an adjunct, but when Polyak reported that the gene encoding POX (PRODH), was one of a small group robustly induced by p53, the central tumor suppressor gene (14), it became apparent to us that POX and the proline cycle functioned primarily in metabolic stress situations, and that this was the area upon which we focused during the last decade.

2.3. Proline oxidase

It was fortuitous that the resurgence of interest in metabolism coincided with our studies of proline during the last decade. This was especially true for cancer research. Although important insights have been gained from advances in genomics and identification of oncogenes, suppressor genes and signaling networks, a critical recognition was that many of these networks accommodate the bioenergetic requirements for survival or to supply the necessary building blocks for rapid proliferation (15). An important consideration is the differential metabolic requirements along the timeline of tumorigenesis. Initially, tumor cells are detached from the basement membrane and from their blood supply with resultant hypoxia and nutrient stress. Surviving this period and following angiogenesis, the tumor diverts nutrients for synthesis of cellular constituents and energy is obtained not from oxidative phosphorylation but from glycolysis. In other words, carbons are used for growth rather than oxidized to CO₂ for energy (16). Thus, glycolysis, the conversion of glucose to lactate, is used for energy, whereas acetyl CoA is used for lipid synthesis rather than for entry into the TCA cycle. Glucose, through the pentose phosphate pathway, is channeled into ribose and the formation of ribonucleotides.

Another area which has advanced in the last decade is the exploration of the tumor microenvironment (17, 18). These studies emphasized not only the interaction...
Proline metabolism and cancer

Figure 2. Schematic representation of the functions of proline oxidase (POX) in tumor suppression. It must be emphasized that the gene encoding POX is not a classical tumor suppressor gene. The markedly decreased or absent expression of POX in human tumors is not due to a somatic mutation or hypermethylation. Instead, POX is suppressed by a microRNA, miR-23b*, which is markedly overexpressed in cancer. POX is induced by p53 and PPARgamma, reflecting genotoxic and inflammatory stress, respectively; it catalyzes the transfer of electrons from proline with intervening electron acceptors to reduce oxygen to form ROS (reactive oxygen species). ROS, by a number of mechanisms, increase proliferation, block the cell cycle and initiate apoptosis. Alternatively, alpha-KG, produced from proline by sequential dehydrogenations, destabilizes HIF-1alpha to block its proliferative signaling. These mechanisms suppressing tumors occur in the absence of metabolic stress (hypoxia and nutrient deprivation). Abbreviations are as follows: PPARgamma, peroxisome proliferator-activated receptor gamma; MAPK, mitogen-activated protein kinase; COX-2, cyclooxygenase-2; GADD, growth arrest and DNA damage; PHD, prolyl hydroxylase; HIF, hypoxia inducible factor; VEGF, vascular endothelial growth factor; others are as shown in legend for figure 1.

3. POX AS METABOLIC TUMOR SUPPRESSOR

3.1. Mechanisms characterized in tissue culture

With Polyak’s discovery that PRODH, the gene encoding POX is among a select group of genes rapidly and robustly induced by p53 (14), we and others showed that the increased expression of POX played a role in apoptosis (20-22). Importantly, we showed that the induction of POX generated proline-dependent ROS (20, 23). With the concomitant ectopic expression of SOD1, SOD2 or catalase, we identified superoxide as the specific ROS linking POX to the induction of mitochondrial apoptosis. Although the site or molecular mechanism for the production of superoxide remains undefined in mammalian mitochondria, the direct reduction of O2 by electrons from FADH has been shown using purified recombinant enzyme from Thermus thermophilus, by Tanner and colleagues (24, 25). In our studies, the redox coupling mediated by POX affected a number of pathways central to apoptosis (Figure 2). Yongmin Liu in our laboratory showed that the overexpression of POX downregulated MEK/ERK signaling (26), decreased COX-2 levels (27), decreased beta-catenin and destabilized HIF-1alpha (28). In addition, POX- mediated signaling blocked transition through the cell cycle at the G2-M check point by increasing the expression of GADD and geminin. Almost all these mechanisms required the production of ROS as ectopic expression of SOD2 blocked the proline-mediated effect. The only exception was the destabilization of HIF-1alpha where alpha-ketoglutarate, sequentially produced from proline and glutamate, seemed to augment the prolyl hydroxylation of HIF-1alpha thereby increasing its VHL-dependent proteosomal degradation. Collectively, all these POX-dependent mechanisms observed in tissue culture under conditions of normoxia, and an abundance of nutrients (Dulbecco’s medium), robustly decreased cell growth or mediated programmed cell death (Figure 2).

3.2. POX suppresses tumors in a xenograft animal model

To extend these effects to animals, we developed a xenograft model in immunodeficient mice using DLD-tet-off-POX cells (28). DLD cells are colorectal cancer cells which rapidly form tumors when injected into the flanks of immunodeficient mice (29). The expression of POX can be controlled by giving mice doxycycline in their drinking water. The expression of POX in tumors was shown to be under doxycycline control in vivo. When POX was suppressed, tumors readily formed in all the animals within a few days and these grew to a size requiring euthanasia by the 3rd week. In contrast, when POX was expressed by doxycycline withdrawal, tumors were suppressed and none of the animals developed tumors requiring euthanasia. In fact,
Proline metabolism and cancer

when the study was terminated at the end of the 5th week, less than 25% had palpable tumors i.e. greater than 100 cm. Thus, the inhibitory effects on tumor cell growth were corroborated in an animal model.

3.3. POX is downregulated in human tumors

Although we have demonstrated that POX is a mitochondrial tumor suppressor protein in cultured cells and in a xenograft tumor model, a critical question is whether clinically significant human tumors have overcome the POX-mediated tumor suppressor effect. We obtained 97 frozen tissues representing a variety of human tumors and performed immunohistochemical studies for POX. Normal tissues from the same patient served as controls. These studies showed that 61% of all tumors had decreased expression of POX compared to normal tissues. Strikingly, with tumors of the digestive tract and of the kidney, 78% of the tumors showed markedly decreased expression of POX. With these findings, we focused on the mechanism by which tumors decreased POX expression. We sought somatic mutations and single nucleotide polymorphisms affecting catalytic activity. However, we found no differences between tumor and normal tissues. Thus, PRODH does not satisfy the canonical requisite for a tumor suppressor gene. We also examined POX genomic DNA for hypermethylation of both the coding and promoter regions. However, we found no differences between tumor and normal tissues. Investigators have proposed that microRNAs can inhibit the expression of a tumor suppressor protein, thereby functioning as oncogenes (30, 31), Wei Liu in our laboratory initiated a major effort to investigate the role of miRs in tumors escaping from the suppressor effects of POX.

4. MECHANISM FOR POX DOWNREGULATION IN TUMORS

4.1. Identification of miR-23b*

We first performed computer searches matching sequences in the 3’-UTR region of POX mRNA against possible miR sequences. Using miR microarrays, we sought differences comparing normal renal epithelial cells and human renal tumor cells. We found 5 sequences which satisfied both criteria (32). Using mimic miRs of these 5 sequences, we tested their effect on normal kidney epithelial cells and in DLD-POX cells under conditions of POX overexpression. We found that only miR-23b* treatment yielded a robust decrease of POX protein in Western blots.

4.2. POX and miR-23b* in human clear cell renal carcinomas

To test the clinical relevance of this specific miR, we obtained frozen tissues from 16 clear cell carcinomas with matching normal tissues from the same patient (32). Using both normal and tumor tissues, we performed quantitative PCR for miR-23b* and Western blots for POX protein quantitated by densitometry. POX was decreased and miR-23b* increased in tumors compared to controls. More importantly, the values for POX protein was negatively correlated with miR-23b* levels with a correlation coefficient of -0.75 and a "p" value less than 0.001. In addition, paired tissue sections were analyzed for mir-23b* by in situ hybridization and for POX by immunohistochemistry. Corroborating the findings on qPCR and Westerns, we found an increase in miR-23b* and a decrease in POX in tumors versus their normal controls. Based on these studies, we concluded that miR-23b* is the causative mechanism for the loss of POX expression in human renal tumors.

4.3. Restoration of POX-mediated tumor suppression by inhibition of miR-23b*

The reversal of the miR effect in silencing POX as a tumor suppressor protein would be a critical test for the role of miR-23b*. The inhibitory antagonist with complementary base pairs was studied in tissue culture as a proof of principle. First we showed in DLD-POX cells that treatment with miR-23b* could blockade the increase in ROS and suppression of cell growth mediated by POX expression. More importantly, in renal tumor cells where POX expression was low and miR-23b* levels were high, treatment with the inhibitory antagonist of miR-23b* increased both ROS and the percent of cells undergoing apoptosis in a POX siRNA-inhibitable manner. Thus, the use of miR-23b* antagonists may provide a method to restore POX expression in tumor cells to mediate its tumor suppressive effect (32). We are currently developing delivery systems for the antagonist optimized in tissue culture to test our hypothesis in animal models. In summary, these studies on POX and PRODH, the gene encoding POX, have not only elucidated the mechanism by which POX blocks the cell cycle and mediates apoptosis, but also introduced the possibility of reactivating the apoptotic effect of POX by inhibiting a specific miR as a therapeutic strategy in human tumors.

5. ALTERNATIVE MECHANISMS FOR REGULATING POX

5.1. PPARgamma

Paralleling our effort to establish POX as a p53-initiated signaling mechanism for apoptosis and the role of miR-23b* in its regulation of POX at the level of translation, we also undertook studies to broaden our understanding of the regulation and functions of POX. Pandhare and Cooper, using a POX-promoter luciferase construct, tested a number of transcriptional factors (33). They found that although some well-known transactivators, e.g. c-jun, c-fos and p65, modestly activated the POX promoter, the most potent was the peroxisome proliferator activated receptor gamma, increasing relative luciferase activity more than 6-fold. We established that PPARgamma was a transcriptional activator by both electrophoretic mobility shift assays (EMSA) and by chromatin immunoprecipitation (ChIP) assays. The finding that PPARgamma, in addition to aforementioned p53, regulated transcription of the POX gene was of considerable interest. PPARgamma is activated not only by prostaglandins, but also by thiazolidinediones, important hypoglycemic drugs used for treatment of type 2 diabetes. In the context of apoptosis, the PPARgamma-dependent generation of ROS was shown to be POX-dependent in colorectal cancer cells, and also
Proline metabolism and cancer

Figure 3. Role of POX signaling under conditions of metabolic stress. With glucose deprivation, AMPK is activated to increase POX expression whereas oxidized ligands from oxLDL bind to PPARgamma to induce POX. The ROS from POX activates autophagy for survival by upregulating beclin expression and to initiate autophagy as demonstrated by the conversion of LC3 I to LC3 II in the formation of autophagosomes. Abbreviations are: oxLDL, oxidized low-density lipoprotein; LC3, microtubule associated protein 1 light chain 3; others are as shown in legend to figures 1 & 2.

shown by others in non-small cell carcinoma cells of the lung (34). Additionally, investigators have found that oxidized lipids are also ligands for PPARgamma (35, 36). The protein-protein interactions with PPARgamma with a number of co-activators are also important. This includes RXR and PGC-1, thereby linking the activation of POX to paradigms for regulating metabolism (37). Furthermore, PPARgamma plays an important role in inflammation by inhibiting the expression of inflammatory cytokines and to direct the differentiation of immune cells towards an anti-inflammatory phenotype (38).

5.2. Physiologic ligand for PPARgamma upregulates POX

Pharmacologic ligands for PPARgamma were potent inducers of POX expression, but we sought a physiologic/pathophysiologic ligand to activate this versatile regulatory system. A link between PPARgamma and lipid metabolism has been demonstrated by others. Additionally, oxidized lipids can function as ligands and activators of PPARgamma signaling (35). Thus, we wondered whether oxLDL would increase POX expression by this pathway. Indeed, Olga Zabirnyk found that oxLDL, but not unoxidized LDL, stimulated POX expression by activating the POX promoter (39). The blockade of the effect by knockdown of PPARgamma showed that it was a necessary link in this signaling cascade. Interestingly, unlike the activation of PPARgamma by thiazolidinediones, the downstream effect of the induction of POX did not involve initiating apoptosis. Instead, autophagy was induced in a POX-dependent fashion.

5.3. PPARgamma, POX and autophagy

Autophagy, literally “self-eating,” has been identified as a mechanism by which cellular components and organelles are engulfed and degraded in autophagosomes (40, 41). The sequential expression of specific genes induced the formation of these autophagosomes. Endosomes containing extracellular materials fuse with these autophagosomes and finally with lysosomes which contain the necessary degradative enzymes. Although previously considered as Type 2 programmed cell death, autophagy more likely generates nutrients for survival under conditions of nutrient depletion. It may also be that the degradation of damaged organelles maintains survival by preventing deterioration of cellular function.

Autophagy as a mechanism for survival is regulated mainly through the mTOR network (42). This system senses metabolic stress and nutrient depletion and responds to halt protein synthesis and cell growth. Interestingly, both rapamycin which can directly bind to mTOR to prevent formation of TOR complex 1 and aminoimidazole carboxamide ribonucleotide (AICAR) which activates AMPK, markedly activated POX activity (43). Decreasing levels of glucose activated AMPK, and upregulated POX. As a complement to these findings with metabolic stress, we tested the effects of hypoxia. As expected, hypoxia activated POX, but the mechanism for this activation was not dependent on HIF-1alpha. Instead, POX activation with hypoxia also occurred through an AMPK-mediated mechanism. As found with oxLDL and PPARgamma, the effects of hypoxia activated both autophagic and apoptotic mechanism (44). Under these conditions of metabolic stress, however, POX was not necessary for apoptosis; knockdown of POX did not block apoptosis. Presumably other pathways could stimulate the apoptotic cascade. But POX was a necessary mediator of autophagy (Figure 3). Knockdown of POX markedly inhibited the induction of beclin 1, the conversion of LC3-I to LC3-II and prevented the formation of autophagosomes (44). Thus, we have shown that POX plays an important role in both apoptosis and autophagy. These POX-mediated mechanisms can be important for tumor cell survival or they can provide an approach for adjunctive cancer therapy. But whence comes the proline?

6. AUTOPHAGY AND SURVIVAL

6.1. POX and nutrient deprivation in autophagy

Under conditions of adequate blood supply – i.e. for normal cells with attachment to basement membrane on the one hand, or invasive or metastasizing
tumor cells following neovascularization, on the other – glucose is the main source of energy. Of course, the shift from oxidative phosphorylation in the former to glycolysis in the latter has been described in detail. Additionally, glutamine serves not only as a source of carbons for conversion to other amino acids, but also as a source of amino groups for de novo nucleotide synthesis. Under certain circumstances, glutamine is also used for energy (45). But both glucose and glutamine are delivered by the circulation, and under conditions of inadequate blood supply with hypoxia, these nutrients would not be available. In fact, oxygen diffuses through tissues more readily than either glucose or glutamine. With inadequate blood supply, nutrient deficiency is perhaps more marked than even hypoxia, and under conditions where amino acids are deficient for the tumor cell as sensed by mTOR, glutamine will also be deficient. Under conditions of nutrient depletion and metabolic stress, which nutrients are available to the tumor cell in its microenvironment?

6.2. Proline and glutamine

Some investigators have suggested that lipids are used as alternative source for bioenergetics during nutrient depletion and metabolic stress (46). Certainly fat deposits are available in certain tumors and the enzymes of lipolysis are upregulated during nutrient deprivation. With autophagy, much of the cellular structure contains lipids that can be used for energy. However, if glucose and glutamine are in short supply, tricarboxylic acid cycle intermediates would be depleted. A source of anaplerosis is necessary for supplying the oxaloacetate necessary for condensation with acetyl CoA to enter the TCA cycle as isocitrate. We propose that proline and/or hydroxyproline can be that source. alpha-Ketoglutarate can be derived from proline and glyoxyxlate from hydroxyproline. For the latter, malate synthase condenses acetyl CoA and glyoxyxlate to form malate. This hepatic enzyme in rats has been found to be markedly induced with starvation (47).

6.3. Proline and hydroxyproline in anaplerosis

Researchers interested in the tumor microenvironment have emphasized the role of stroma or extracellular matrix (ECM), surrounding invasive tumor cells (48). Detached from its source of blood supply, tumor cells as well as stromal cells activate and/or release a variety of matrix metalloproteinases (MMPs) which are primarily collagenases (49). Thus, collagens are degraded and the resultant peptides are recognized by uPARAP/Endo180 receptors and taken up into endosomes which fuse with lysosomes and autophagosomes where these peptides are degraded by cathepsins and proteases (50). However, dipeptides containing either proline or hydroxyproline in the carboxyl terminus cannot be degraded by the usual dipeptidases because the peptide bond contains a tertiary amine; these imidopeptides require their own hydrolytic enzyme, prolylase (51), which releases free proline and hydroxyproline. The complete hydrolysis of collagen would release primarily proline, hydroxyproline and glycine (percentage of residues: 17%, 17.9% and 33%, respectively. Proline is released to participate either in protein synthesis or to be metabolized by POX to produce ROS for signaling or as a source of energy as previously mentioned (see above). Glycine, of course, is used for protein synthesis, but it can also be cleaved to be a methyl donor through tetrahydrofolate and also generates energy in the form of NADH (52). Whether the NADH is bioenergetically contributory under conditions of nutrient deprivation has to be investigated. An important consideration is the metabolism of hydroxyproline. Hydroxyproline is not recycled for protein synthesis. Instead, it is sequentially metabolized to glyoxyxlate and pyruvate (53). This is of interest since glyoxyxlate from hydroxyproline (also from glycine) can condense with acetyl CoA to form malate as part of the glyoxyxlate cycle (54), the degradation of collagen may be an anaplerotic mechanism for the TCA cycle and the metabolism of fatty acids. This mechanism is at present hypothetical, but preliminary experiments suggest that it occurs.

7. METABOLISM IN THE TUMOR MICROENVIRONMENT

7.1. Glucose and glutamine in tissue culture

The seminal work elucidating the mechanisms for the Warburg Effect has emphasized the importance of switches in glucose and glutamine metabolism (10-12, 45). Indeed, on first inspection, it appears as if all the adaptations for survival and bioenergetics can be satisfied by adaptation in the metabolism of these two substrates. However, the insights gained from tissue culture studies may not fully explain what occurs within the tumor microenvironment. First, lymphoma and leukemia cells were used in the majority of these studies. These cells are not subject to the same vascular isolation imposed on solid tumors. Furthermore, tissue culture conditions were developed to optimize the growth of malignant cells. Thus, Dulbecco’s MEM contains 4500 mg/L (450 mg/dL) glucose whereas circulating venous glucose in humans is 100-120 mg/dL Glutamine, on the other hand, 0.5-1.0 mM in venous plasma, whereas in Dulbecco’s MEM it is 4.0 mM. Thus, tissue culture medium contains a surfeit of nutrients and does not reflect the interaction of the developing solid tumor within its microenvironment.

7.2. Degradation of extracellular matrix by matrix metalloproteinases (MMPs)

The question arises -- Although collagen can be a reservoir for proline for protein synthesis, does activation of MMPs with increased collagen degradation provide free proline and hydroxyproline under stress conditions? MMPs are certainly activated under conditions of metabolic stress (hypoxia, nutrient deprivation) (55, 56). They are also upregulated accompanying inflammation (57) and tumorigenesis (58). Direct evidence that collagen is degraded under these conditions comes from a variety of studies. An early study by Marian & Mazzucco showed that dermal collagen was markedly degraded accompanying the two-stage skin tumor model so often used to study carcinogenesis (59). More recently, Glunde and colleagues, using secondary harmonic generation for visualizing collagen I fibers, showed that hypoxia is accompanied by a decrease in collagen I fibers (60). Although few
Proline metabolism and cancer

Figure 4. Timeline showing the role of POX in tumorigenesis. The timeline is simplified into 3 stages – stress, survival and proliferation. During the period of genotoxic or inflammatory stress, POX is induced to generate proline-dependent ROS which can initiate both apoptosis and autophagy. During this period, nutrients (glucose and glutamine) are not perturbed. Malignant transformation occurs at the end of this period. The survival period is characterized by inadequate blood supply with resultant hypoxia and depletion of circulating substrates. Notably, cells are deprived of glucose and glutamine. During this period, POX is upregulated by AMPK, and proline (as well as hydroxyproline) is supplied by activation of matrix metalloproteinases to degrade extracellular matrix (ECM) in the microenvironment. ECM is predominantly collagen, rich in proline and hydroxyproline. POX can use proline as a source of ATP, metabolic intermediates, and ROS to signal autophagy. In the proliferation phase, vascularity has been restored and there is adequate oxygen and substrates. Tumors are “addicted” to glucose, generate ATP by glycolysis and use glucose and glutamine as precursors for cellular constituents necessary for growth. Under these conditions POX is downregulated through miR-23b* to minimize its apoptotic effects and to conserve proline for protein synthesis and the formation of extracellular matrix.

7.3. MMPs and tumorigenesis

The role of MMPs in degrading extracellular matrix has been extensively studied in tumorigenesis and was initially proposed as a mechanism for removing physical barriers thereby promoting tumor invasion and metastasis (17). The initial enthusiasm culminated in the testing of MMP inhibitors in a number of clinical trials (18). However, these trials were universally unsuccessful leading to a re-evaluation of this therapeutic strategy. Inhibitors of MMPs may be a two-edged sword. Beneficial, i.e. tumor inhibitory effects, as well as tumor promotion effects have been identified with activation of MMPs. Furthermore, the release of various cytokines bound to ECM, TGF-β, in particular, has been invoked as the mechanism by which MMPs exert their effect (63). Recently, the degradation and remodeling of collagen have been found to affect tumor behavior by a mechano-regulatory mechanism (64). Throughout these investigations of the influence of MMPs in the tumor microenvironment, little attention has been paid to the proline (and hydroxyproline) released and the effect of their metabolism on the tumor cell. Recent discoveries will stimulate investigators to pursue research in this area. In fact, it is likely that insights gained from the understanding of glucose/glutamine metabolism will find parallels in collagen degradation and the metabolism of proline and hydroxyproline in the microenvironment of tumors.

8. TUMORIGENESIS AND A METABOLIC TIME LINE

Nevertheless, the diverse mechanisms which stimulate POX expression and activity, and the many downstream effects of POX signaling may seem paradoxical, at first inspection. POX can mediate survival mechanisms with autophagy, but also functions as a tumor suppressor and a mediator of programmed cell death (5-7). Perhaps this paradox may be best understood in the context of a time line beginning with metabolic and genotoxic stress leading to malignant transformation (7), followed by mechanisms for tumor survival and finally mechanisms for rapid growth. Features of this model are simplified and represented in Figure 4. Pre-transformation stress includes inflammation, DNA damage, apoptosis and anoikis. With transformation, there is release from growth constraints and loss of suppressor activities. However, once the tumor has become invasive and detached from its blood supply, hypoxia and nutrient depletion ensue, which activate compensatory mechanisms including autophagy for survival. When neoangiogenesis has supervised with adequate vascularization and ample supply of oxygen and nutrients, the tumor exhibits the Warburg effect with
Proline metabolism and cancer

decreased oxidative phosphorylation and increased glycolysis and glutaminolysis. Substrates are channeled or diverted into pathways for building cell mass – for producing proteins, lipids and nucleotides. Within this time line of carcinogenesis, the changes in POX become more understandable. During the period of stress and transformation, glucose metabolism is basal, but POX may be activated with both inflammatory and genotoxic stress to blockade the cell cycle for repair or to initiate apoptosis. During the stage of invasion with accompanying hypovascularity, glucose and glutamine depletion accompany hypoxia. POX is induced by AMPK, downregulation of mTOR as well as by PPARgamma-dependent mechanisms to activate autophagy for survival. Proline metabolism can be a source of ATP as well as a supply of alpha-ketoglutarate for anaplerosis. However, when angiogenesis has restored blood supply with adequate oxygen and nutrients, glucose and other nutrients are channeled into cell mass allowing rapid proliferation of tumor cells. At this stage, POX is suppressed by miR-23b^*

9. PERSPECTIVES

It is exciting that studies in proline metabolism are on the cusp of important discoveries impacting on a number of research and clinical areas. A few of these areas deserve mention here. First, the partner of POX in the proline cycle, P5C reductase, is being intensely studied by several groups of researchers with important discoveries. The identification of cutis laxa with PYCR1 deficiency (8) and the characterization of three isozymes of P5C reductase (9) have major implications for understanding the importance of proline metabolism. The recent emphasis of the biochemistry and molecular biology of mitochondria will attract renewed effort in defining the molecular mechanisms for electron transport by POX and the role of POX in the generation of signaling ROS. Hydroxyproline oxidase is another area which deserves attention. Unlike proline, hydroxyproline is not proteinogenic and is degraded to pyruvate and glyoxylate (1). Thus, it may contribute to the metabolic adjustments made by the Warburg effect without depleting the pool of proteinogenic amino acids. Finally, the recent emphasis on the microbiome is a reminder that many of the interesting regulatory effects of proline were first identified in prokaryotes and in parasites (1). The possible interaction between the organisms of the microbiome and mammalian host tissues may have metabolic consequences, either beneficial or harmful. It is, indeed, an exciting time for proline.

10. ACKNOWLEDGEMENTS

This review was adapted from the Keynote Address given at the 3rd International Symposium on Proline Metabolism held in Lincoln, Nebraska, November 7-9, 2010. Organizers of the meeting were Donald Becker, U. of Nebraska-Lincoln, and John Tanner, U. of Missouri-Columbia. The work was supported by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research. This project also has been funded in part with Federal funds from the National Cancer Institute, NIH, under contract no. HHSN27612080001. The content of this review does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. government. We thank Dr. Anna Maciag for her reading of the manuscript.

11. REFERENCES


Proline metabolism and cancer


34. KH Kim, YS Cho, JM Park, SO Yoon, KW Kim and AS Chung: Pro-MMP-2 activation by the PPARgamma agonist, ciglitazone, induces cell invasion through the generation of ROS and the activation of ERK. FEBS Lett, 581, 3303-3310 (2007)


Proline metabolism and cancer


44. W Liu, et al. The role of proline oxidase in the prosurvival autophagic response to hypoxia (In preparation)


Proline metabolism and cancer

**Abbreviations:** POX: proline oxidase, PRODH: proline dehydrogenase, PPARgamma: peroxisome proliferator-activated receptor gamma, MMPs: matrix metalloproteinases

**Key Words:** Apoptosis, Autophagy, Metabolic stress, Survival, Review

**Send correspondence to:** James Phang, Metabolism and Cancer Susceptibility Section, Laboratory of Comparative Carcinogenesis, Center for Cancer Research, NCI-Frederick, Frederick, MD 21702, Tel: 301-846-5367, Fax: 301-846-6093, E-mail: phangj@mail.nih.gov

http://www.bioscience.org/current/vol17.htm