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EXPERIMENTAL STUDY

The ubiquitous expression of pyruvate carboxylase among human prostate tumors

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ABSTRACT
Pyruvate carboxylase (PC) is a mitochondrial enzyme catalyzing the ATP-dependent reaction of pyruvate prolongation with bicarbonate ion to oxaloacetate. The synthesis of oxaloacetate by PC, an intermediate of the Krebs cycle, is recently recognized as a significant anaplerotic reaction that supports the biosynthetic capability, growth, aggressiveness, and even viability of several cancer cell types. PC expression was confirmed in several types of cancer cells and tumors. To evaluate the possibility that prostate tumor-forming cells are also exploiting the anaplerotic role of PC, we applied immunoblotting analysis to estimate its presence. Our results revealed that PC is present among the lysate proteins derived from prostate cancer and benign prostatic hyperplasia samples. The expression of PC in cells of prostate tumors and benign prostatic hyperplasia supposes that PC could facilitate the formation of oxaloacetate *in situ* and enhance the autonomy of their biosynthetic metabolism from the availability of extracellular substrates by increasing the cellular anaplerotic capability *(Tab. 1, Fig. 1, Ref. 30)*. Text in PDF *www.elis.sk*
KEY WORDS: pyruvate carboxylase, prostate cancer, cancer metabolism, anaplerosis.

Introduction

The growth and division of tumor-forming cells depend on appropriate substrates for sustaining energy metabolism and biomass formation processes (1). In this respect, the Krebs cycle reactions significantly contribute to both processes (2–5). The consumption of the Krebs cycle intermediates could challenge the viability of cells (2), and therefore their loss has to be replenished by anaplerosis (6, 7). Only a few reactions possess the anaplerotic role in cellular metabolism. In addition to glutamine catabolism, which is considered a prominent anaplerotic process in cancer cells (8–11), pyruvate carboxylation has been recognized as being necessary for sustaining the growth and viability of several types of cancer cells even in the presence of glutamine (5, 5, 12–20).

Pyruvate carboxylase (PC; pyruvate: carbon-dioxide ligase (ADP-forming), EC: 6.4.1.1) is a biotin-containing enzyme catalyzing an ATP-dependent addition reaction of bicarbonate anion to pyruvate by synthesizing oxaloacetate (21). Even though the gene for PC apoenzyme is nuclearly encoded, the enzymatically active holoenzyme is present in mitochondria. The expression of an enzymatically active PC is a complex process regulated at several levels in a cell-type-specific manner (20).

By considering the varying availability of exogenous substrates and capacity of cancer cells to metabolize them, glucose seems to be the primary precursor for endogenous pyruvate. In this respect, the synthesis of oxaloacetate from glucose-derived pyruvate by PC might provide the cancer cell metabolism with a higher degree of autonomy and independence from the supply of extracellular substrates, including glutamine (15, 19, 22).

The *in vitro* experiments on several types of cultured human cancer cells revealed that the anaplerotic process profoundly impacts their cellular metabolism, biosynthetic capacity, growth, and viability (12, 13, 15, 17–19, 23–26). The importance of the anaplerotic process for supporting the growth and survival of cancer cells might be exploited to develop new diagnostic tools and therapeutic approaches. Therefore, knowledge about the frequency of PC expression *in situ* among different cancer types might be beneficial. Indeed, PC expression in several types of tumor-forming cells and cancers has already been confirmed. Even though prostate tumors belong among common types of cancers in males, the aspects of their anaplerotic metabolism remain insufficiently studied. To test the possibility that PC could also be expressed in human prostate tumors, we used the immunodetection-based approach, i.e., the dot-blot method.

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Tab. 1. Characteristics of the patients with prostate tumors.

	Prostate cancer		CRPC	BPH
	GS ≤ 7	GS > 7		
Number	4	4	4	4
Age (years)	69.3±3.6	71.8±4.6	74.3±4.6	71.8±0.7
Gleason score (%)	6.5±0.2	8.3±0.1	9.0±0.1	ND
PSA (ng/ml)	13.9±2.5	20.7±5.7	32.9±15.7 *	2.2±0.3
Pathological stage (number)				
pT1/pT2	1	0	0	ND
pT3/pT4	3	4	4	ND

GS – Gleason score; CRPC – castration-resistant prostate cancer; BPH – benign prostatic hyperplasia;* $p < 0.05$ vs BPH

Material and methods

The experimental protocols were approved by the Ethics Committee of the Jessenius Faculty of Medicine CU in Martin under the code FK 1255/2013. Informed consent was obtained from all subjects of this study. The selected characteristics of the patients, who were donors of prostate tumor specimens, are in Table 1.

Processing of tumor samples

The prostate tumor samples were collected just after surgical dissection and processed to lysates as already described (Kmeťová Sivoňová et al, 2021). Before further analysis, tumor lysates were stored at –20 °C. The content of proteins in lysates was estimated by modifying the Bradford's method (27) using a commercially supplied solution and bovine serum protein as a standard.

Dot blot analysis

The lysates derived from the prostatic cancers were used to detect pyruvate carboxylase expression by the dot-blot method. Two μg of lysate proteins were spotted on nitrocellulose membrane and let to be air-dried at RT in the dark. The free binding sites on the membrane were blocked by incubating the membrane in a blocking solution (BS) consisting of tris-buffered saline solution (TBS; 19.8 mM Tris, 136 mM NaCl, with pH adjusted to 7.6) supplemented with Tween 20 (0.08 %) and bovine serum albumin (2 %) at room temperature for 30 min. After that, the membrane was incubated in 10 ml of the mixture of rabbit antibodies against pyruvate carboxylase diluted 1:200 in BS at 4 °C overnight. Subsequently, the membrane was washed three times with TBS supplemented with Tween 20 (TBS-T) for 10 min, and then the membrane was probed with a solution of anti-rabbit IgG molecules conjugated with horse-radish peroxidase (1:200) in BS for 2 h. After rinsing the membrane 3x in TBS-T for 10 min and 1x in TBS, the membrane was soaked in SuperSignal West Pico Chemiluminescent Substrate solution (Thermo-Scientific), and the chemiluminescent signal was recorded by Chemidoc XRS system (Bio-Rad Laboratories, USA).

The obtained chemiluminescent signals for PC was quantified with the Image Studio Lite version 5.2 software (LI-COR Biotechnology, Inc., USA)

Statistical analysis

The results are presented as mean ± SEM. The student's t-test was used to test the difference between two prostate cancer groups with Gleason score either equal and below or above 7. One-way analysis of variance with *post hoc* comparisons by Student-Newman-Keuls test was carried out to test for the differences among all tested groups of either brain or prostatic tumors. The differences between data sets were considered statistically significant if the *p* values were lower than 0.05. The statistical analysis was performed with the software GraphPad Prism version 9.0.2 (GraphPad Software, USA).

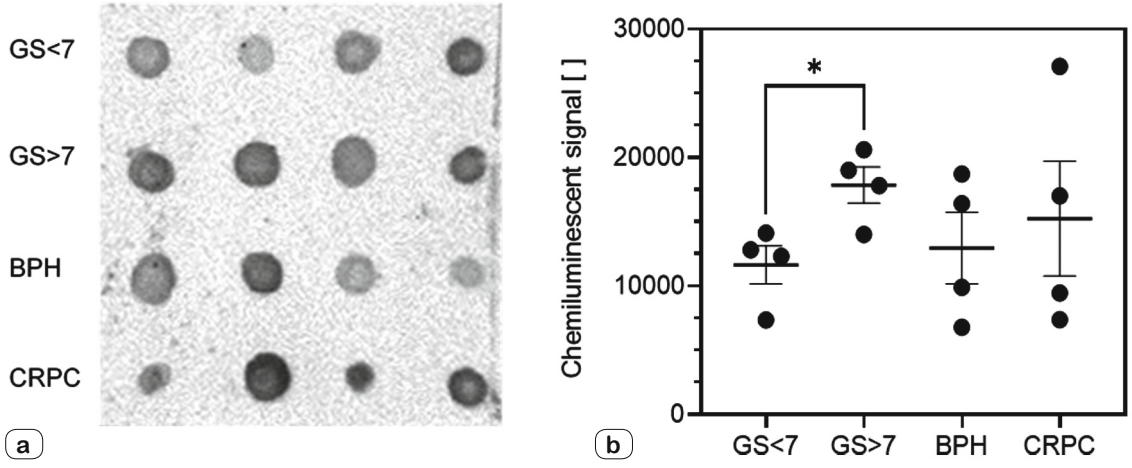

Fig. 1. The dot-blot analysis of PC expression of lysates in prostate tumors. The lysates of prostate cancer samples with Gleason score either ≤ 7 (GS ≤ 7) or above 7 (GS > 7), benign prostatic hyperplasia (BPH), and castration-resistant prostate cancer (CRPC) were spotted on the nitrocellulose membrane and probed with rabbit antiserum against PC and subsequently with affinity-purified anti-rabbit IgG molecules covalently linked to horse-radish peroxidase. The chemiluminescent signal was recorded (a), and its intensity was quantified (b).

Results

The suggested important roles of PC in supporting the metabolism and features of cancer cells in culture and animals evoke the question to which extent PC is distributed among human tumors *in vivo*. The presence of PC among the proteins in lysates from prostate tumors (Tab. 1) obtained after the surgical resection was investigated by immunoprobing analysis. The samples of prostate tumors were subdivided into four groups: with the values of Gleason score equal and lower to 7 or above 7, castration-resistant prostate cancer, and benign prostatic hyperplasia.

The immunoprobing of the lysates derived from prostate tumors with rabbit antiserum against PC followed by affinity-purified anti-rabbit IgG molecules covalently linked to peroxidase (POD) provided the chemiluminescent signal originating from all tested samples (Fig. 1a). The quantification of the obtained chemiluminescent signal shows that PC levels within tested groups vary to a different extent (Fig. 1b). The analysis of variance among the estimated values for the chemiluminescent signals in groups of different types of prostate tumors did not reveal the significant differences among them. However, a comparison of the mean values of the chemiluminescent signals between two groups of prostate cancers with Gleason scores either below up to equal or above seven by student t-test revealed that they differ significantly ($p=0.027$).

Discussion

Here we present the data revealing the presence of PC among human prostate tumors.

The performed dot-blot analysis with a set of antibodies applied on the tumor lysates belongs to the group of immune-detection methods that represent a valuable tool for qualitative assessment of selected antigen presence. The primary antibodies applied in the study were initially derived against the bovine orthologue of PC (28). Due to a high degree of homology in primary sequences between bovine and human PC orthologues (20, 29) and capability to recognize only PC among several types of protein lysates derived from different human cells and tissues (12), the used antibodies can be considered suitable also for the dot-blot analysis performed on human prostate tumor lysates.

Several study models had been exploited to study and obtain the results dealing with the altered expression of genes during carcinogenesis and their function in cancer metabolism. Indeed, PC expression and function were studied on several types of cultured cancer cell lines of human origin and animal models (5, 5, 12–20). However, the complexity in the regulation of PC expression might be limiting with respect to the translational aspect of those results. Therefore, the test performed on human prostate tumor samples allows for more reliable confirmation of PC gene expression *in situ* and deduction of its metabolic and cellular functions.

PC is a biotin-containing enzyme, whose enzymatic activity is regulated at several levels (20). In addition to its regulated expression, which is cell-specific, allosteric modulators and posttranslational modifications affect the overall PC activity (20, 22). Exploited immunoblotting method, based on the application of PC-specific antibodies, can confirm the presence of PC apoenzyme. The biotinylation of PC apoenzyme to holoenzyme by holocarboxylase synthase is necessary to express an enzymatically active form. Since the recently performed experiments in our laboratory have shown that i) the positive immunoblotting signal of PC detected among lysate proteins from several types of human cancer cell lines and brain tumors co-occurs with the enzymatically active form of PC, ii) treatment of astrocytoma lysate with streptavidin-sepharose prevented the detection of immunoblotting signal with anti-PC antibodies, while therefore pointing to the fact that PC is present in cancer cells as holoenzyme with covalently linked biotin and its apoenzyme form is, if present, only at an undetected level (Gondáš et al, in press). By analogy, we might assume that the holoenzyme of PC is also predominating in prostate cancers. In addition, the enzymatic activity of PC could be allosterically regulated, i.e., ADP stimulates the activity of PC by several folds (20). Therefore, the actual contribution of PC to the cellular metabolism of prostate tumor-forming cells remains to be assessed.

During carcinogenesis, several molecular changes, including the transformation of cellular metabolism, happen. In this respect, the transforming cancer cells alter genes' expression to support the alternations in metabolism underlying their adaptation to the composition of their microenvironment. Besides supplying the energy demands, the cancer cell metabolism also supports their growth and division by the anabolic process. The limited availability of substrate molecules in the cellular microenvironment and glucose preference by cancer cells suggests the emerging role of the Krebs cycle as a source of precursors for biosynthetic processes (2, 30). In this respect, the anaplerotic processes seem to be essential to prevent the collapse of mitochondrial metabolism (6, 7).

Conclusion

The expression of PC by the cells in prostate tumors is an essential prerequisite for exploiting its anaplerotic capability. Anaplerosis is a process crucial for sustaining the energy metabolism and biosynthetic capacity of cancer cells with an impact on their growth, dividing capacity, or even viability. Due to the fundamental role of anaplerotic metabolism in sustaining the cellular processes, it has become the object not only of research interest but is also the target of several therapeutical anti-cancer strategies. Indeed, the approach based on the possibility to inhibit glutaminolysis in cancer cells is already exploited in therapy against several types of human tumors. In this respect, the knowledge about the expression of PC in human prostate tumors may be helpful not only as information for a better understanding of anaplerotic metabolism in prostate tumors but might also be helpful for designing novel anti-cancer strategies that could be directed against the key enzymes participating in the cancer cell metabolism.

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