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EVALUATION OF METABOLIC STATUS OF CANCER CELLS BY HISTOENZYMATIC AND IMMUNOHISTOCHEMICAL ANALYSIS

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ABSTRACT. In the past few years the medical treatment of malignant diseases has steadily increased in scope and importance. Monitoring the effects of this treatment is very important both of study of the influence on the cell metabolism. Mitochondria are subcellular organelles, whose function is to produce ATP through the oxidative phosphorylation system. Alterations in respiratory activity appear to be a general feature of malignant cells. The aim of the present study is to analysis and evaluation of metabolic status of cancer cells by use histoenzymatic techniques to determine the activity of the ATPase, LDH, and immunohistochemical analysis of cytochrome *c* release, both in tissue samples were obtained before the antineoplastic treatment and after antineoplastic treatment.

Keywords: cytochrome *c*, cancer cells, ATPase, LDH

INTRODUCTION

The subcellular organelles mitochondria have the main function to produce ATP through the oxidative phosphorylation system. Mitochondria are also essential in the processing like carbohydrates, amino acids and fatty acids. In addition, mitochondria are semi-autonomous organelles that perform an essential function in the regulation of cell death, signaling and free radical generation (Czamecka A.M et al, 2007).

Recent evidence suggests that oxidative metabolism may have a key role in controlling cancer growth. More than 80 years ago, Otto Warburg suggested that impaired oxidative metabolism may cause malignant growth. This assumption, later known as Warburg's hypothesis, has been experimentally addressed for many decades. Oxidative phosphorylation, is one of the key ways a cell gains useful energy. It is the set of the metabolic reactions and processes that take place in organism cells to convert biochemical energy from nutrients into adenosine triphosphate (ATP), and then release waste products (Ristov M., 2006).

Aerobic respiration requires oxygen in order to generate energy (ATP). Although carbohydrates, fats, and proteins can all be processed and consumed as reactant, it is the preferred method of pyruvate breakdown from glycolysis and requires that pyruvate enter the mitochondrion in order to be fully oxidized by the Krebs cycle. The product of this process is energy in the form of ATP, by substrate-level phosphorylation by NADH and $FADH_2$.

Glycolysis is a metabolic pathway that is found in the cytoplasm of cells in all living organisms and is anaerobic. The process converts one molecule of glucose into two molecules of pyruvate, and makes energy in the form of two net molecules of ATP (Ristov M, 2006, Kondoh, H., 2009).

Alterations in respiratory activity appear to be a general feature of malignant cells. (Carew, J.S., Huang, P., 2002) The cancers are always associated with the alteration of the energetic cellular metabolism, connected to the reducing function of the mitochondria (Cruce M. et la 2004, Cucuianu, M. et al, 1998, Gagvadze V. et al, 2008).

Warburg pioneered the research on alterations of the mitochondrial respiratory chain in the context of cancer and proposed a mechanism to explain how they evolved during the carcinogenic process. In his series of landmark publications, he hypothesized that the key event in carcinogenesis could be the development of an injury to the respiratory machinery, resulting in compensatory increases in glycolytic ATP production (Ristov, M., Cuezva, J.M., 2009, Scatena R et al 2009).

Malignant cells produce their ATP through glycolytic mechanisms rather than through oxidative phosphorylation. In benign tumors the rate of aerobic glycolysis is only 1/3 that in malignant tumors (Amuthan, G. et al 2001, Kim J., Dang Ch.V, 2006).

Pelicano H. et al. specify that the cancerous cells are more dependent from glycolysis for the generating of the ATP, even then when oxygen is found in abundance in the cell, the glycolysis from the cancerous cells being an aerobe glycolysis (Pelicano, H. et al, 2003).

In experimental condition it was demonstrate that the oxidative phosphorylation inhibitors depleted ATP and induced apoptosis (Eguchi, Y., 1997, Gagvadze V. et al. 2008, Olinici C.D., 2010).

The mechanisms through which the tumoural cells change their metabolism are not known both how these changes affect the answer at antineoplastic medicines (Kim J., Dang Ch.V, 2006).

The discovery of the regulating mechanism of these metabolic pathways would stock or deliver important information for the diagnostic and for the antineoplastic therapy (Gagvadze V. et al, 2008, Kim J., Dang Ch.V, 2006).

Our study is a contribution in elucidate these pathways and proposed the following of the cellular

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metabolic activity with the help of some histoenzymatic and immunohistochemical analysis.

MATERIALS AND METHODS

Adenosine-triphosphatases (ATPases) are a class of enzymes that catalyze the decomposition of ATP into ADP and a free phosphate ion. This dephosphorylation reaction releases energy, which the enzyme (in most cases) harnesses to drive other chemical reactions that would not otherwise occur. This process is widely used in all known forms of life.

Lactate-dehydrogenase (LDH) is a NAD(P) dependent cytoplasm enzyme catalyzes the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD+ (Koukourakis MJ et al., 2005).

Cytochrome c is an electron transporting protein that resides within the intermembrane space of the mitochondria, where it plays a critical role in the process of oxidative phosphorylation and production of cellular ATP. Following exposure to apoptotic stimuli, cytochrome c is rapidly released from the mitochondria into the cytosol, an event which may be required for the completion of apoptosis in some systems. Cytosolic cytochrome c functions in the activation of caspase 3 and ICE family molecule that is a key effector of apoptosis.

Our study monitored of energetic metabolism by ATPase activity, as well as the activity of lactate dehydrogenase (LDH) for marking out the glycolytic processes in the cytoplasm, both of the release of cytochrome *c* from intermembrane space of mitochondria into the cytosol and activation the apoptosis.

Mammary tumor tissue samples have been used, which were obtained by biopsy following the surgical interventions at the County Clinical Hospital of Oradea (Romania), between 2008-2009. Tissue samples were obtained before the antineoplastic treatment and after six months of antineoplastic antiangiogenetic treatment with bevacizumab.

Histoenzymatic techniques are based on highlighting the reaction product, as a result of the action of a specific enzyme upon a specific substrate. When the reaction product is soluble, in order to prevent it from diffusing from the reaction site, it requires to be made insoluble employing a capture reaction. In order to be observed under a microscope, the reaction product must be stained employing a visualization reaction, carried out by adding chromogen substances to the incubation medium or sometimes after the incubation.

The main stages for obtaining a histoenzymatic sample are similar to those in classical histology, but there are two specific stages: incubation and visualization (Cotrutz, C-tin et al., 1994, Muresan E et al, 1976).

The organ fragments are immersed in special containers, in isopentane cooled with liquid nitrogen and completely frozen. In order to obtain the best histoenzymatic samples we sectioned using the cryotome at 20 μm.

We used Wachstein - Meissel method for determination of adenosine triphosphatase (ATPase) activity. ATPase is an enzyme that breaks the ATP into energy and phosphate. ATP is a result of mitochondrial oxidative phosphorylation, rarely a result of glycolysis in normal cells. ATPase liberates by ATP hydrolysis inorganic phosphate that in the presence of lead forms a coloured precipitate. Areas with enzymatic activity present brown-black granules. (Cotrutz, C-tin et al., 1994, Muresan E et al, 1976)

For determination LDH, we use Hess-Scarpelli-Pearse method, H+ is accept from a tetrazolium salt which is reduce and results a formazan dye, areas with enzymatic activity present dark blue granules. (Cotrutz, C-tin et al., 1994, Muresan E et al, 1976, Cucuianu, M. et al, 1998)

Cytosolic cytochrome c was determined with immunohystochemical Cytochrome c Ab-2 mouse monoclonal antibody kit, used standard immunohystochemical protocols with LSAB2/HRP (labelled streptavidin-biotin/horseradish peroxidase).

The samples were analysed by light microscopy. Results were evaluated by accepted semiquantitative method for percentage expression. (Van Beek-Harmsen B.J., Van der Laarsen W.I., 2005)

In microscopy images were randomly chosen 10 different fields, the semiquantitative evaluation of enzyme activity or immunohistochemical reaction was performed as follows:

- = abesnce of enzyme activity;
-/+ = low enzyme activity;
+ = increased enzyme activity;
++ = very intense enzyme activity.

RESULTS AND DISCUSSIONS

Before treatment ATPase distribution is heterogeneous, with approximately equal distribution of areas with low and intense enzyme activity and almost half of the analyzed fields have high activity (Figure 1) after antiangiogenetic treatment shows decrease ATPase activity, areas with low activity doubled compared to begenning of treatment (Figures 2 and 3).

LDH enzyme shows a slight increase activity in the malignant cells during the antiangiogenetic treatment, with decrease of low enzyme activity and increase on intense and very intense enzyme activity fields in the tissue samples obtained after the antineoplastic treatment (Figures 4, 5 and 6).

With reference to cytochrome *c* immunoreactivity occur the most significant changes. In approximate six months of antiangiogenetic treatment have dissapeared the negative immunoreactivity areas (Figure 7).

In these cells carries a strong apoptotic activity due to release cytochrome c in cytosol. The microscopic images can be seen live with nuclei intracellular apoptotic cells, apoptotic cells with extracellular

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nuclei, dead cells devoid of cytoplasmic content (Figures 8 and 9).

CONCLUSIONS

The antineoplastic treatment leading to a significantly decreased synthetized ATP quantity, a condition that leads to the death of the tumor cell, being deprived of the energetic macromolecules that are necessary for a normal functioning.

The ATP is not a result of the mitochondrial activity and of the citric acid cycle linked to oxidative phosphorylation but most likely is the result of glycolysis.

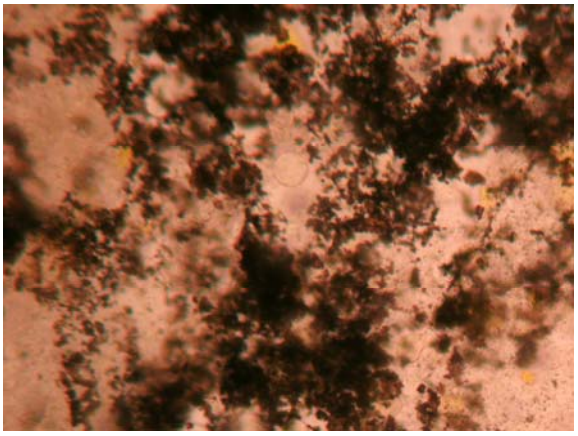

Fig. 1 ATPase activity before treatment (x200)

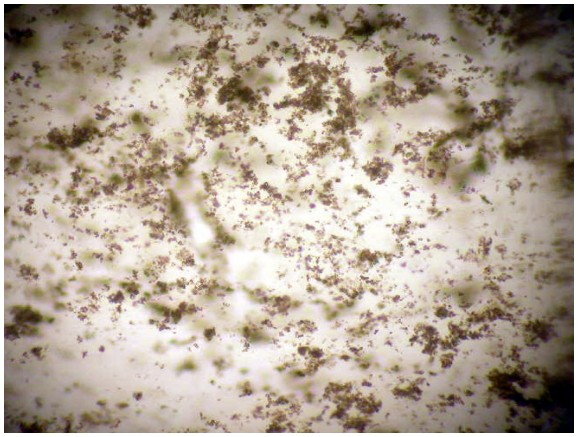

Fig. 2 ATPase activity after treatment (x200)

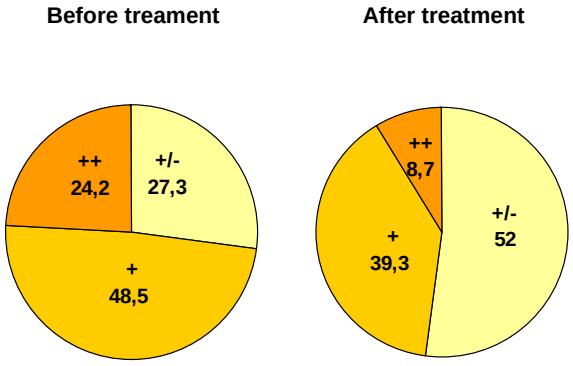

Fig. 3 Semiquantitative evaluation of ATPase activity

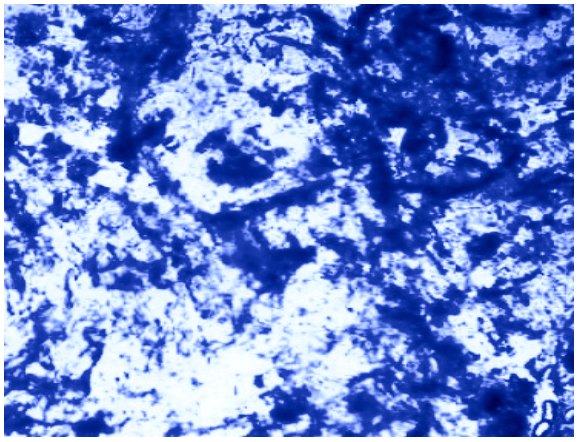

Fig. 4 LDH activity before treatment (x200)

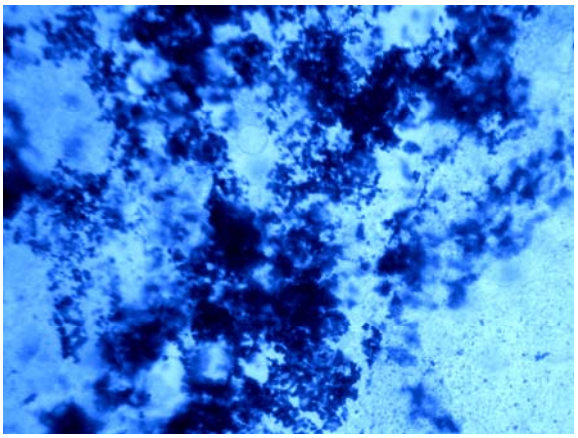

Fig. 5 LDH activity after treatment (x200)

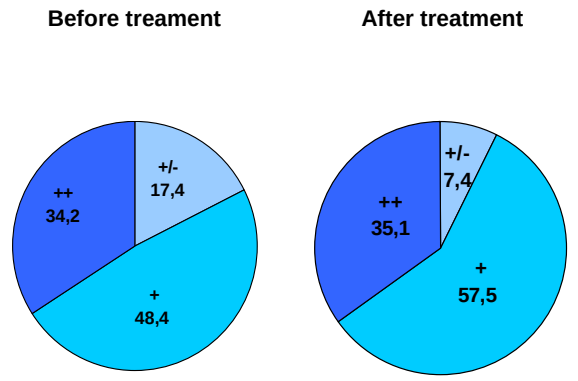

Fig. 6 Semiquantitative evaluation of LDH activity

The recent researches done in biological and oncological fields have demonstrated that the cellular metabolism and mainly the energetic metabolism is affected of the effects of antineoplastic medicines, these operating at the transcription and translation level of the phosphorylation protein chain (Kondoh H. et al, 2009; Manash KP et al, 2006).

More ATP is a key factor in the activation of executive caspases which are created in the final phase of apoptosis. The discovery of the regulating mechanisms of these metabolic pathways would deliver important information for the diagnostic and antineoplastic treatment (Scatena R. et al, 2009).

As the level of ATP determines the entrance of the cell in apoptosis, (Eguchi Y. et al, 1997; Czarnecka et al., 2007), and the apoptosis process needs a significant quantity of ATP, the quantity of ATP synthetizes with glycolysis is enough to initiate apoptosis.

This study demonstrated the metabolic status change with activation the apoptosis process and decreased synthesis of ATP, so efficacy as antiangiogenetic treatment.

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