EXTENDED ABSTRACT

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Cytobiochemical biomarkers of the state of mitochondria in Humans. I. A new assessment of Warburg effect by the ratio of lactate dehydrogenase to succinate dehydrogenase activity in lymphocytes as a distinct biomarker of pronounced differences between leukemia, norm and myopathy in young patients

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Abstract

The paper describes a method for determining alterations in the state of mitochondria in children suffering leukemia and myopathies using the original cyto-BIO-chemical (CBCH) method. The method gives a unique solution to the actual problem of the fundamental medicine - the evaluation of mitochondria in Humans without intervention into the organism or damaging isolated organelles. It was shown that under immense changes in the state of the organism, differences in the state of mitochondria are strongly manifested using CBCH biomarkers, such as activity of succinate dehydrogenase (SDH), lactate dehydrogenase (LDH) and the LDH/SDH ratio proposed by us as an indicator of the Warburg effect (WEF). This parameter markedly increases in case of leukemia and decreases in case of myopathies.

In addition to these biomarkers, here we demonstrate previously unknown changes in the appearance of lymphocytes of surviving during development of staining. The changes were observed in case of diseases and absent in lymphocytes from healthy individuals. These changes differ, when incubated with different mitochondrial substrates. They were the most pronounced upon addition of lactate that is considered as an oncogenic metabolite. All these new biomarkers are available and applicable in clinical trials.

List of abbreviations: *CBCH – cyto-bio-chemical, SDH - succinate dehydrogenase, SUC – succinate, LDH - lactate dehydrogenase, LAC – lactate, WEF - Warburg effect, NBT - nitroblue-tetrazolium, NBR - nitroblue reduction, ISC – isocitrate, MAL – malonate.*
Short title: Mitochondria in Humans. Warburg effect.

Introduction

The paper refers to the actual area of modern personalized medicine – the search for biomarkers of the state of mitochondria in Humans. Despite the urgency of this problem, there is no adopted solution to it. A traditional biochemical method of mitochondrial isolation from tissue sample causes deterioration of mitochondrial association into a network, which destroys a fine physiological regulation of mitochondrial functions. Although the assessment of mitochondrial parameters often

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demonstrates significant changes; in many cases they are just artifacts caused by experimental conditions of isolation and not physiological state of mitochondria in vivo. For a long time, our group has been developing a method allowing detection of fine changes in mitochondrial parameters without damaging mitochondria. The method is based on cytochemical method of very mild fixation of mitochondria within lymphocytes as a smear using a special thoroughly chosen incubation media. It allows preserving the state of mitochondria in vitro close to their native state in vivo and detecting functional changes with much higher accuracy than by other methods. The method was described in the patent and publications (Kondrashova et al., 2009; Zakharchenko et al., 2013). Here we use biomarkers for detection of mitochondrial changes under severe pathologies such as leukemia and myopathies in children.

Materials and methods

Studies were carried out on blood samples taken from a group of 5 to 7 patients within a short period. The patients were surveyed in the above-mentioned clinics in compliance with ethical standards. Blood samples were taken from 2 or 3 healthy donors simultaneously or sometimes at the same time on the next day. Periodically, a group of 3 to 5 healthy people and 1 or 2 patients were examined. Simultaneous examinations of small groups under the same conditions give more accurate comparative characteristics of the state of examinees, than those obtained by a comparison of average values obtained in large groups under varying conditions and at different days.

CBCH method for determination of dehydrogenase activity.

1) Preparation of a standard thin blood smear by a commercially available device V-Sampler "Vision" (Austria) without using anticoagulant, all in 7-10 sec.
2) Fixation of smears with 60% acetone, 10mM HEPES, pH 5.2-5.4, for 30 sec.
3) Incubation of the sample for an hour at 37°C in medium (pH 7.2±0.05) containing 1.22 mM nitro-blue-tetrazolium (NBT) dye, 125mM KCl, 10mM HEPES, substrates and inhibitors: for SDH activity - 5mM SUC (succinate); for SDH activity regulated by ISC - 5mM SUC + 5mM ISC (isocitrate) ; for LDH activity - 5 mM LAC (lactate) + 5 mM MAL (malonate) + 0.5 mM NAD.
4) Staining of nuclei with 0.5% neutral red for 8 min.
5) Computer quantification of formazan staining in microscopic images (100 blood lymphocytes per sample).
Obtained data are statistically significant due to a large number of analyzed objects, $p<0,05$ (Kondrashova et al., 2012). As standard deviations are too small, they are not shown in Figures (Kondrashova et al., 2012).

Results and discussion

1. Pronounced differences in the LDH, SDH activities and WEF in lymphocytes between leukemia patients, normal individuals and patients with myopathy.

Figure 1 shows typical examples of enzyme activity in the comparable groups. LDH activity is particularly useful for the study, since it is significantly higher than that of the most powerful respiratory enzyme – SDH. In order to fit the measurement result of both enzymes in the same figure, we have introduced two scales. The LDH scale is five times compressed as compared with the one for SDH. Measurements of SDH activity were performed without or with ISC, which allows detection of hyperactivation or suppression of SDH by attenuation or stimulation upon addition of ISC respectively

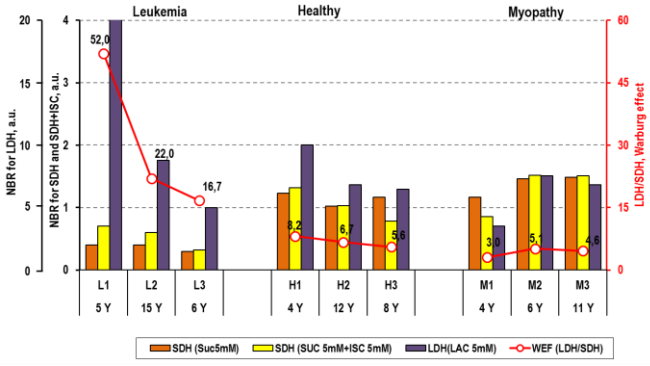

Figure 1. Biomarkers of glycolysis (LDH), respiration (SDH) and Warburg effect (LDH/SDH) in young patients suffering from leukemia (L) and myopathy (M), compared with healthy (H) individuals of similar age, measured by nitro-blue tetrazolium reduction (NBR). The examinees fall into groups as follows: L - 22, H – 25 and M - 9.

The absence of apparent effect of ISC on SDH activity shows that SDH functions within the normal range of

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activation. We saw that SDH activity in leukemic patients is decreased, while LDH is much higher than in healthy individuals. Since these changes are reciprocal, the LDH/SDH ratio is much higher in leukemic patients. We propose to use it as a new indicator of the Warburg effect, which can be measured in Humans. Its increase, as shown by us in cases of leukemia, is in conformity with the published data on the Warburg effect in cancer cells, assessed by other methods.

In contrast to leukemia, in case of myopathies SDH activity is slightly higher than normal, while LDH is considerably reduced, as compared to healthy individuals. As a result, the LDH/SDH ratio is lower than normal. Such a reduction in the Warburg effect was not encountered in the literature. However, we can explain this effect based on the new understanding of the role of aerobic glycolysis for cancer cells, actively developed by Thompson (Ward et al., 2012). According to this idea, aerobic glycolysis is not a weakness, but an advantage of cancer cells. It provides more intensive flow of metabolites for the growth and division of cells, than respiration does. However, there is no need in powerful energy supply for intensive cell growth. Thus the decreased glycolysis revealed under conditions of myopathy, may reflect a weakening of recovery biosynthetic processes. This understanding is in good conformity with the known state of cells under myopathies, characterized by general weakening of functions and structures. This shift in the state of tissues is also observed by the appearance of lymphocytes from patients with myopathy, which is described in the next section.

2. Appearance of lymphocytes as a visual biomarker of their state.

In addition to the changes in the enzyme activity in children with leukemia, we detected strong alterations in the appearance of lymphocytes and monocyte-like cells during incubation for one hour with various metabolites (oxidation substrates), especially in a lactate-containing medium. Such modified cells do not appear in smears obtained from healthy people. That is why we call them pathological. The difference between patients and healthy individuals is well observed through the appearance of cells stained in the lactate-containing medium.

Leukemia cells are markedly enlarged. Their nuclei are intensely coloured. Myopathy cells are less enlarged. At the same time, they appear thinned compared to the norm. Their membranes have numerous villi that look like droplets of flowing cytoplasm.

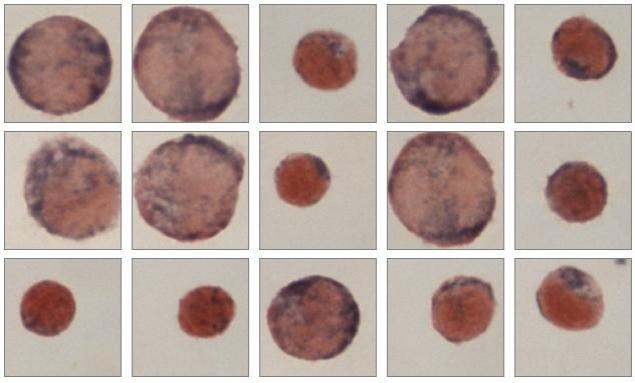
Thus, the surviving lymphocytes somehow change their appearance. The changes differ depending on the metabolite (oxidation substrate) added. Addition of lactic acid gives the most prominent differences.

Lactic acid is a pathogenic metabolite, in particular – oncogenic (Kristen et al., 2016). Its level increases in tumors due to enhanced glycolysis. Recently, a strong regulatory effect of lactate on the immune cells, which leads to increased tumor growth, was described (Ward et al., 2012). The immune mechanism of this regulation has been studied. We assume that the changes in the appearance of cells, that we have found, are associated with these regulatory alterations.

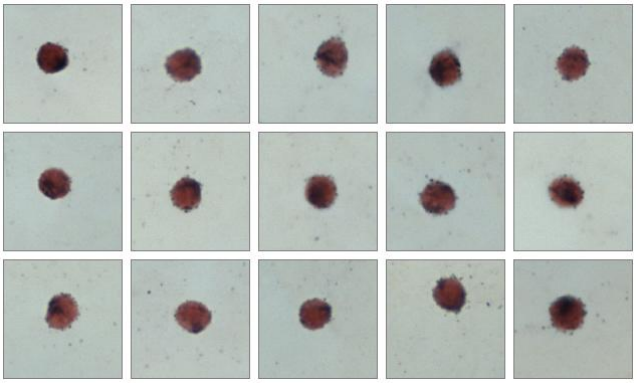
Visualization of such clearly modified cells helps to distinguish normal and pathological situations, as well as is important for determining the severity of the disease, in practical medicine

In particular, assessment of the percentage of pathological lymphocytes can show the capacity of biological resistance of a certain patient, i.e. the severity of the disease suffered.

Leukemia



Healthy



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Myopathy

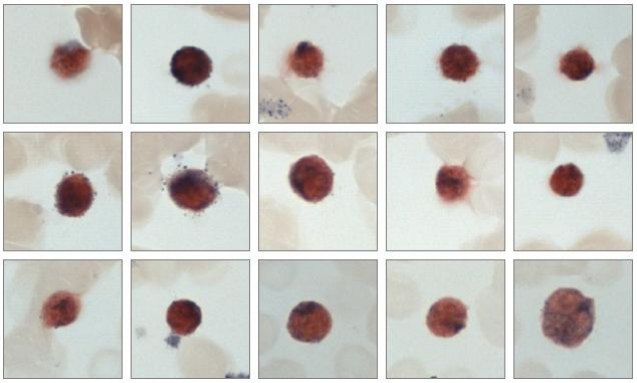

Figure 2. Images of Human glass-adhered lymphocytes oxidizing Lactate. Images were collected using an oil immersion lens (100x/1.25). LDH activity is registered by blue staining of formazan. Red staining with neutral red is used for nuclei visualization.

Conclusion

Assessment of LDH, SDH and their ratio as a measure of WEF using CBCH method is a sensitive biomarker of changes in the state of mitochondria under severe diseases: leukemia and myopathy.
Visual evaluation of lymphocyte appearance after incubation with substrates is useful for rapid detection of changes in the state of mitochondria in medical practice.

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