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A New Glance on Energy Metabolism Investigation in Cultured Cells

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Abstract

The aim of the study was to analyze the input of cytosolic and oxidative pathways of energy metabolism in ATP production of cultured cells by using FLIM and routine biochemical techniques. Fluorescent imaging of endogenous cofactors NADH and FAD demonstrated a more pronounced oxidative redox status of fibroblasts compared to tumor cells and significant differences in metabolic processes in which FAD is involved. Analysis of glucose and lactate content and absorption showed that tumor cells not only absorb glucose more intensively from the environment, but also use it more intensively during anaerobic glycolysis. Lower energy efficiency of glycolysis and FAD oxidative path and greater energy consumption is the reason for the lower concentration of ATP in tumor cells. Presumably, the prevalence of glycolytic metabolism in tumor cells could be largely determined by their hypoxic reprogramming through the PI3K/AKT/mTOR signal pathway. The results of the study suggest that correlation between intracellular consumption of glucose and cytosolic concentration of NADH may contribute to the characteristic of energy metabolism state of cultured cells and serve as the biosensor of malignant cell transformation.

Keywords

Metabolic Imaging, Glycolysis, ATP, Tumor Cells of HeLa Kyoto

1. Introduction

An organism needs energy all the time, which is obtained from various catabolic processes, such as glycolysis, fatty acid oxidation, TCA cycle [1] [2] [3]. During catabolic pathways, electron pair is removed from substrates and transferred to

NAD+ or FAD+, this generates reduced coenzymes NADH+ and FADH2– the main substrates for the mitochondrial electron transfer chain (ETC), where ATP is synthesized in the reaction of oxidative phosphorylation (OXPHOS) and NADH oxidizes to NAD+ and FADH2 to FAD+. The intracellular NADH и FAD could be used as the natural biomarkers of the oxidation-reduction reactions, bioenergetics of the cells and tissues, particularly during the tumor growth. NADH and its oxidized form NAD+ take part in the regulation of energy transformation, Ca++ homeostasis, gene expression, oxidative stress, aging and apoptosis. The pool of cytosolic and mitochondrial NADH/NAD+ differs [4] [5]. FADH2/FAD+ is the next redox-pair which takes part in tissue respiration in all eukaryotic cells. FAD+ mainly connects with mitochondria and oxidative way of phosphorylation. To sum up, the ratio of cofactor (redox ratio) reflects the cell's overall metabolic activity and redox status of the cells [6] [7]. Glycolysis, also known as Embden-Meyerhof pathway, is the oldest known pathway and is present in most cells. It provides a metabolic cross-road and is tightly coupled with energy production, especially during many pathological states, including hypoxia, cell growth, etc., when the fortification of redox homeostatic systems is observed [8].

Alteration of cellular energy metabolism is a principal feature of tumor. Tumors reprogram pathways of nutrient acquisition and metabolism to meet the bioenergetics and redox demands of malignant cells. Recent work has demonstrated remarkable flexibility in the specific pathways activated by tumor cells to support these key functions [9].

The example of a reprogrammed metabolic pathway in cancer is the Warburg effect [10]. Glycolysis is a physiological response to hypoxia in normal tissues, but Otto Warburg observed that tumor slices and ascites cancer cells constitutively take up glucose and produce lactate regardless of oxygen availability, an observation that has been seen in many types of cancer cells and tumors. The increase in glycolytic flux allows glycolytic intermediates to supply subsidiary pathways to fulfill the metabolic demands of proliferating cells to active growth and proliferation and adaptation to heterogeneous microenvironment conditions [11]. At the same time, a growing number of papers show that many cancer cells retain mitochondrial function and use oxidative phosphorylation as the primary way of generating energy [12]. It is assumed that in order to maintain high metabolic activity, cancer cells co-ordinate the consumption of nutrient substrates, primarily glucose [13]. Some cancer cells can reversibly switch between glycolysis and oxidative metabolism, depending on the environmental conditions [14]. It is suggested that cancer metabolism can serve as an indicator of efficiency of anticancer treatment and be used for better understanding the mechanisms of action of anticancer drugs [15].

The cell culture HeLa is widely used for the investigation of cancer metabolism [16].

It is well known that the microenvironment also plays the key role in the pro-

gression, invasion and metastasis of the tumors. Fibroblasts, pericytes, lymphocytes, macrophages regulate the interrelation between stromal and cancer cells by modulation tumor microenvironment. It is showed that these cells could also change their metabolism activity in tumor progression [17].

In our study we re-examine peculiarities of energy metabolism in relation to the current concepts of cancer metabolism as being intimately linked to alterations of redox status, glucose consumption and lactate production, using the modern and routine technique of investigation. Mltiphoton fluorescence lifetime imaging (FLIM) is a promising non-invasive technique for a large number of biological applications. FLIM delivers the highest time resolution and the best lifetime accuracy or photon efficiency [18]. The use of the FLIM method has been widely demonstrated in oncology for not only basic research on cell cultures and animal tumor models but also for clinical imaging of tissue samples from patients [19]. Data about metabolic alterations in cultured cells and tissue samples are important for diagnostic, monitoring the progression of cancer, the development of anticancer drugs therapy.

2. Material and Methods

The cell line of human cervical cancer of HeLa Kyoto and fibroblasts isolated from human skin is used. Fibroblasts were isolated at N.K. Kol'tsov Institute of Developmental Biology of RAS from skin samples removed as a result of primary treatment of the operating field in cosmetic operations in healthy donors with their informed consent according to the protocol approved by the local ethics committee. The cells were cultivated in the DMEM environment (PanEco, Russia) with the addition of glutamine (1%), 10% embryonic veal serum, penicillin-streptomycin (50 micrograms/ml). Cultivation was carried out in vials (25 cm^2) in the environment of 5% of CO_2 at 37˚C and humidity of 85%. Subcultivation was carried out three times a week after the monolayer attained the confluence of ~70%.

Experimental samples were air-conditioned environment, cell lysate and cells planted in cultural cups with glass bottoms. The air-conditioned environment was obtained by cultivating cells in full cultural environment for 72 hours in CO_2 incubator. As a control, a cultural environment without cells was used which was also left in an incubator for 72 hours. For obtaining lysates the tumor cells with 3 - 4 passages, fibroblasts with 4 passages were used. The cells were washed with Versen's solution (PanEco, Russia), removed from vials using a solution of 0.25% of trypsin (PanEco, Russia) and centrifuged at 100 g for 5 minutes. The supernatant liquid was drained, and the deposited cell suspension was lysed with a 5-fold freeze in liquid nitrogen.

For fluorescent microscopy, the cells were put on FluoroDish (World Precision Instruments, the USA) cups in amount of 80 thousand cells per cup 24 hours before the DMEM life experiment without phenol red (Life Technologies, the USA).

The metabolic status of the cells was assessed using two-photon fluorescent microscopy cofactors: the reduced nicotinamide adenine dinucleotide NADH and oxidized flavin adenine dinucleotide FAD.

Fluorescent microscopy was performed on the LSM 710 laser scanning microscope (Carl Zeiss, Germany) equipped with the Chameleon Vision II femtosecond laser (Coherent, the USA) and the FLIM module with time resolution for registration of fluorescence (Becker and Hickl GmbH, Germany). The images were obtained using the C-Apochromat W Korr water immersion objective with 40x magnification and a numerical aperture of 1.3. For the two-photon excitation of NADH fluorescence the wavelength of 750 nm was used and fluorescence was registered in the range of 500 - 550 nm. The fluorescence of the FAD was excited at the wavelength of 900 nm and received in the range of 450 - 490 nm. The power of exciting radiation is 6 mW. The time of registration of fluorescence while obtaining fluorescent images with time resolution was about 300 s. During microscopic shots the cells were located in XL multi S Dark LS mini incubator (PeSon GmbH, Germany) at 37˚C and 5% of CO_2. The intensity of cofactor fluorescence and the FAD/NADH redox ratio was calculated in the ImageJ program (National Institutes of Health, the USA). Redox-ratio was calculated by deducting the background (the signal from the area of the image that did not contain cells) and dividing the signal of the intensity of FAD fluorescence by the intensity of NADH fluorescence. FLIM images were processed in the SPCImage program (Becker and Hickl GmbH, Germany). The attenuation curves of NADH and FAD fluorescence were approximated by the biexponential model. On the basis of the fluorescence attenuation analysis the short (t1) and long (t2) components of life time and their percentage contributions, a1 and a2 were calculated, respectively.

Concentrations of glucose, lactate, ATP, protein were determined by spectrophotometric method (spectrophotometer UV mini 1240, Japan) using Vital kits (AO "Vital Development Corporation").

The statistical analysis was carried out with the help of STATISTICA10, "AnalystSoft" programs.

3. Results

3.1. Metabolic Imaging of Tumor Cells and Fibroblasts

The intensity of NADH fluorescence in tumor cells was statistically significantly higher (26 RU) than in fibroblasts (22 RU), while the intensity of the FAD signal is much higher in fibroblasts (26 RU) compared to tumor cells (10 RU) (**Figure 1**). Accordingly, the total redox ratio of FAD/NADH was significantly higher in fibroblasts compared to tumor cells (1.1 vs. 0.4) indicating a shift in the redox status of fibroblasts towards more oxidized state.

Measurement of life time of NADH and FAD fluorescence in tumor cells and fibroblasts showed that for both types of cells the values of life time were

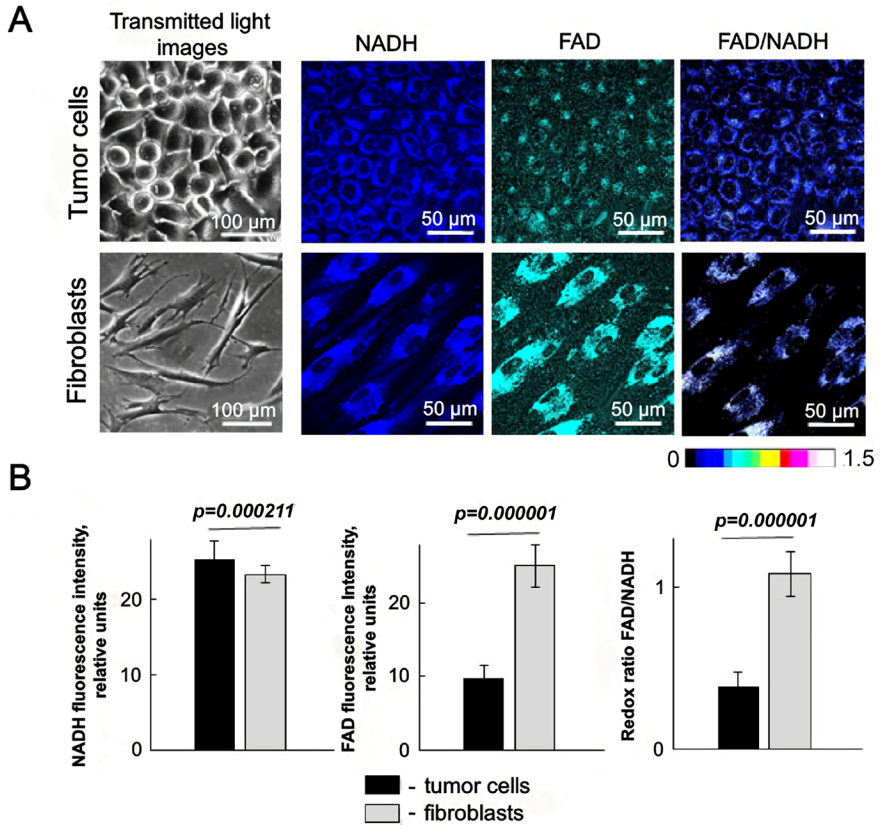

Figure 1. Evaluation of the metabolic activity of HeLa Kyoto tumor cells and human skin fibroblasts by the fluorescence intensity of metabolic cofactors. (A) Representative images of cells in transmitted light. NADH and FAD fluorescence intensity and FAD/NADH redox ratio; (B) Quantitative assessment of the fluorescence intensity of NADH and FAD and the redox ratio.

practically similar: ~0.4 ns (t1) and ~2.5 ns (t2) for NADH and ~0.4 ns (t1) and ~2.6 ns (t2) for FAD, respectively. In the case of NADH, the short life times are characteristic of a molecule in a free form which is associated with glycolysis and the long life times are characteristic of protein-associated forms associated with mitochondrial respiration. In the case of FAD, the short life times are inherent in a molecule in closed conformation and the long life times in open conformation (less associated with protein). The obtained typical lifetime values of fluorescence in associated NADH (t2) indicate that the phosphorylated form of NAD - NADPH, which life time of fluorescence is ~4.4 ns [20], does not make a significant contribution to the total fluorescence of the coenzyme neither in tumor cells, nor in fibroblasts.

Analysis of the relative contributions of free and related forms of NADH showed that the balance of amplitudes a1/a2 (free/bound) in tumor cells and fibroblasts is the same (**Figure 2**). However, the ratio of amplitudes in open/closed FAD conformations, a2/a1, was statistically significantly higher in fibroblasts (0.37) compared to tumor cells (0.44). Since the open conformation of FAD has a higher quantum output of fluorescence, its predominance in fibroblasts could

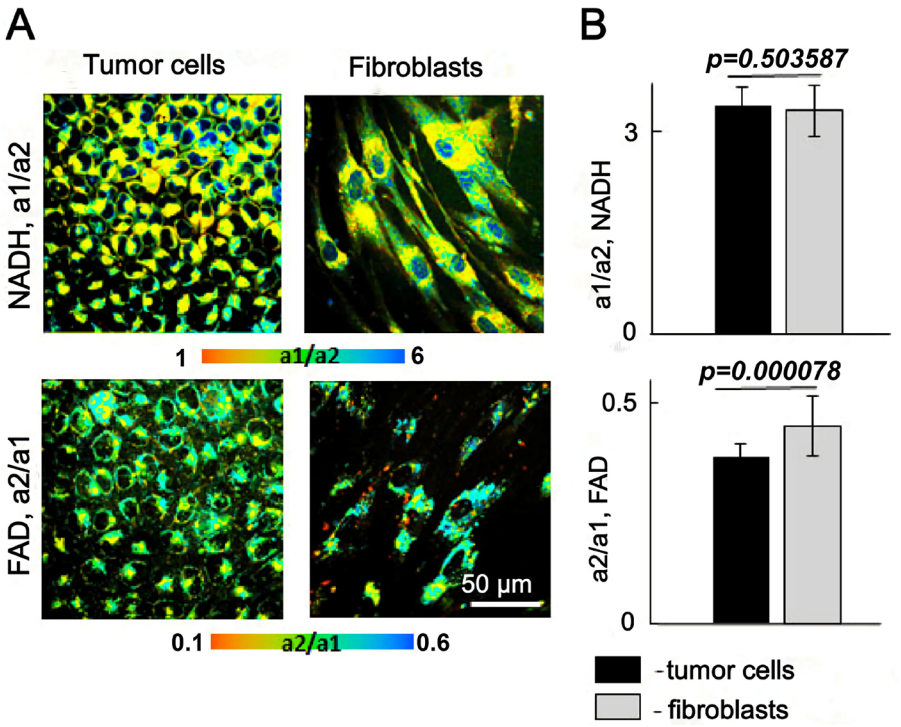

Figure 2. FLIM of metabolic cofactors in HeLa Kyoto tumor cells and human skin fibroblasts. (A) Images of the relationships of the relative contributions of free/related forms of NADH (a1/a2) and FAD (a2/a1); (B) Quantification of a1/a2 NADH and a2/a1 FAD.

contribute to the total emission intensity of this coenzyme along with a higher concentration of molecules.

Thus, the metabolic imaging based on the registration of fluorescence of endogenous cofactors NADH and FAD demonstrated a more pronounced oxidative redox status of fibroblasts and significant differences in metabolic processes in which FAD is involved.

3.2. Biochemical Analysis of Energy and Carbohydrate Exchanges

The glucose content in HeLa Kyoto tumor cells was lower (2.19 ± 0/08 mM/g of cell protein) than in fibroblasts (3.24 ± 0.1 mM/g of protein) (Figure 3(A)).

At the same time, it was found that they consumed glucose from the environment of tumor cells by an order of magnitude more actively (0.14 ± 0.001 mM/g of cell protein compared to fibroblasts, 0.069 ± 0.0003 mM/g) (Figure 3(A)). In the lysates of HeLa Kyoto cells the lactate was present in the amount of 0.66 ± 0.007 mM/g of cell protein, and in the environment of tumor cell cultivation its content increased by more than twice. No lactate was found in fibroblast lysates and their air-conditioned environment (Figure 3(B)).

A decrease in the pool of ATP in tumor cells is found compared to fibroblasts (0.722 ± 0.07 vs. 10.556 + 0.65 mM/g of protein) (Figure 3(C)).

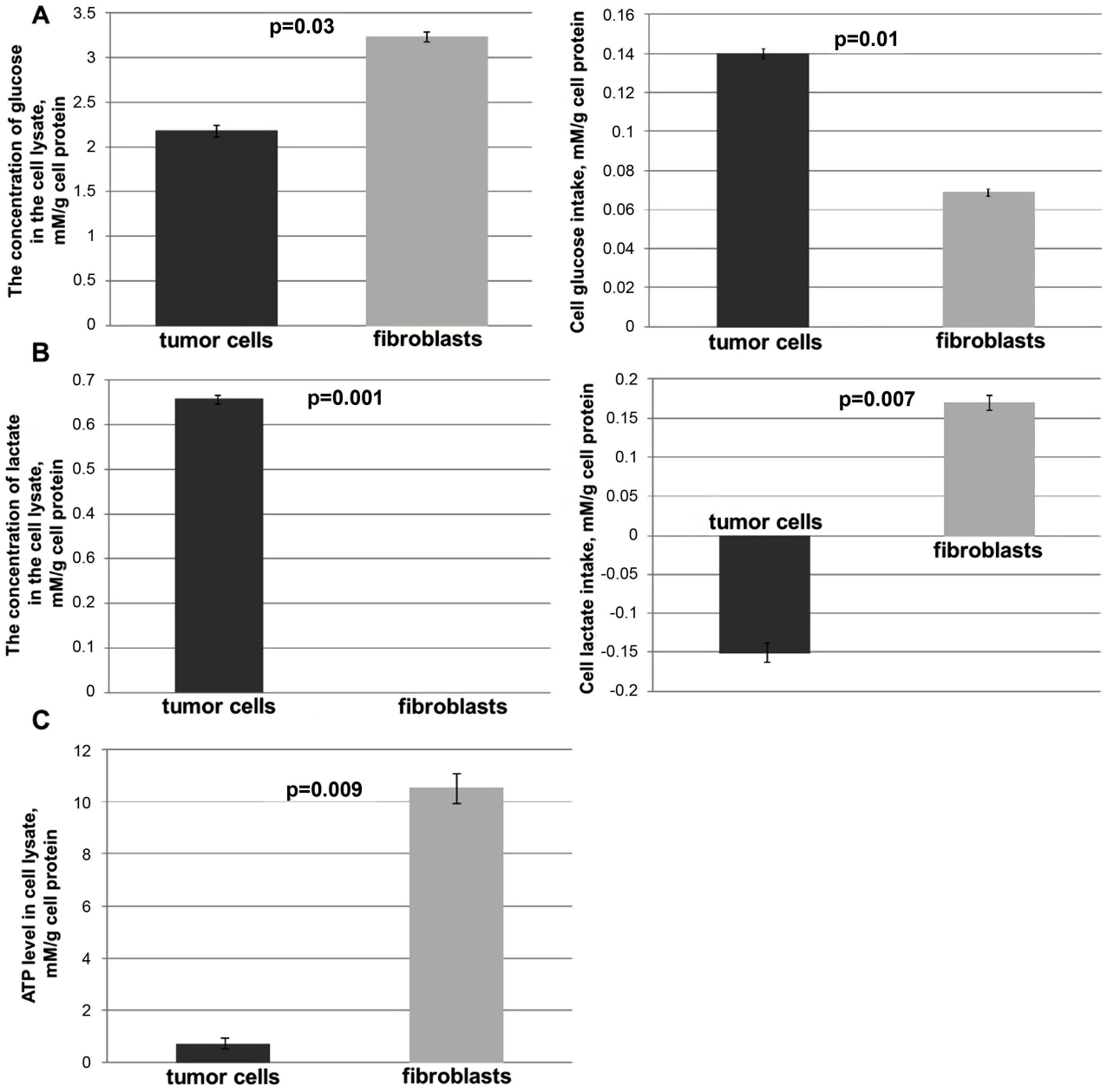

Figure 3. Parameters of carbohydrate and energy metabolism in HeLa Kyoto tumor cells and human skin fibroblasts. (A) The glucose level in cell lysates and its absorption by cells from the medium. (B) The level of lactate in cell lysates and its absorption by cells from the medium. (C) ATP level in cell lysates.

4. Discussion

Among the methods of analysis of the metabolic energy status of cells, increasing attention is paid to the methods of visualizing fluorescence of the cell's own molecules: metabolic cofactors NADH and FAD. The attractiveness of metabolic imagining is primarily due to its non-invasiveness, the lack of need for additional dyes, and the ability to observe metabolic changes in living cells from microscopic level to level of the whole tumor [21] [22]. NAD and FAD cofactors and their reduced equivalents NADH and $FADH_2$ are the key carriers of electrons in

the cell involved in many metabolic reactions, and, above all, in the formation of energy in the form of ATP. NADH is formed during the process of glycolysis and in the TCA cycle. Both cofactors transfer protons and electrons in the mitochondrial electron transfer chain where NADH oxidizes to NAD and $FADH_2$ to FAD. The ratio of cofactor fluorescence intensity (redox ratio) reflects the cell's overall metabolic activity and redox status, and the lifespan of their fluorescence, the state of cofactors, binding to the protein in case of NADH and conformation in case of FAD [23] [24] [25]. If processes other than glycolysis and oxidative phosphorylation do not make a significant contribution to the concentration of cofactors or their condition, the changes in redox ratio and life times of fluorescence are associated only with these two processes.

The decrease in the value of FAD/NADH redox ratio is observed in tumor cells due to the prevalence of cytosolic oxidation pathways. The data we have obtained on the higher levels of FAD/NADH redox ratio in fibroblasts indicate their more oxidized redox status compared to tumor cells which is consistent with the other data [26] [27].

While analyzing the energy metabolism of cells and tissues by the FLIM method, the ratio of free and related forms of NADH is the main indicator. The increase in the relative contribution of the free form of NADH as was shown above (a1) indicates activation of glycolytic metabolism in tumor cells [28] [29] [30].

Reprogramming of energy metabolism is a biological feature acquired during the multistep process of tumor development. This metabolic renewing is crucial in cancer transformation and progression. Mutated oncogenes and tumor suppressor genes are responsible for alterations in metabolic signaling pathways: several oncogenes, such as MYC Proto-Oncogene, Hypoxia Inducible Factor 1 (HIF1), Phosphatidylinositol-4,5-Bisphosphate 3-Kinase (PI3K), AKT Serine/Threonine Kinase (AKT) and Mechanistic Target Of Rapamycin Kinase (mTOR), can stimulate transcription of genes encoding for proteins in the glycolysis and glutaminolysis pathways and induce the loss of p53 functions [31].

Biochemical analysis of glucose and lactate levels and absorption has shown that tumor cells not only absorb glucose more intensively from the environment, but also use it more intensively, which mainly occur during glycolysis. Unlike the fibroblasts, glucose consumed by the Hela cells is active oxidized during anaerobic glycolysis with the production of lactate. We also observed an increase in lactate concentration in the conditioned medium after cultivation of Hela Kyoto cells. Presumably, the prevalence of glycolytic metabolism in the culture of HeLa Kyoto tumor cells, demonstrated through fluorescent metabolic imaging and biochemical studies, is largely determined by their hypoxic reprogramming through the path of PI3K/AKT/mTOR [32].

Anaerobic glycolysis and FAD path of oxidation are energy-less beneficial, which together with a greater consumption of ATP during synthetic processes in tumor cells leads to decrease of ATP in HeLa Kyoto cells. Cancer cells carry out glycolysis and respiration concurrently by shifting the balance between cytosolic and mitochondrial energy producing systems.

The results of the study suggest that correlation between intracellular consumption of glucose and cytosolic concentration of NADH may contribute to the characteristic of energy metabolism state of cultured cells and serve as the biosensor of malignant cell transformation.

Our data suggest that noninvasive, label-free monitoring of the metabolic changes by noting the NADH fluorescence lifetime is also a valuable approach to characterize the responses of cancer cells to anti-cancer treatments and, therefore, to predict the effectiveness of chemotherapy.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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