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Correlation of Changes of Cho – K1 Cells Metabolism to Changes in Protein Expression in Camp Differentiation

Spera R, Vasile F, Pechkova E and Nicolini C*

Nanoworld Institute , Fondazione EL.B.A.Nicolini, Pradalunga, Bergamo, and Laboratory of Nanobiotechnology and Biophysics, Genoa University, Genova, Italy

Abstract

A correlated investigation of cell metabolism and protein expression of Chinese Hamster Ovary cells (CHO-K1) under different growth conditions was performed by ^{1}H nuclear magnetic resonance (^{1}H-NMR), mass spectrometry (MS) and biochemical assays. CHO fibroblasts have shown different metabolic products when grown in different media and when differentiation is stimulated by adenosine 3', 5' cyclic monophosphate (cAMP). In particular, while addition of Fetal Calf Serum causes an increase in the glucose metabolism correlated to changes in lipid composition of the membrane observed by ^{1}H-NMR, differentiation induced by cAMP causes biochemical differences in glucose and lipidic metabolism uniquely correlated both to the specific changes in the composition of nuclear proteins, revealed by mass spectrometry, and to the differences in metabolism, determined by NMR.

Keywords: ^{1}H–NMR; Mass spectrometry; CHO–K1; cAMP; Cell cycle

Abbreviation

CHO-K1=Chinese Hamster Ovary cells; ^{1}H-NMR=^{1}H Nuclear Magnetic Resonance; MS=Mass Spectrometry; cAMP=Adenosine 3',5' Cyclic Monophosphate; RT= Reverse Transformation; Myh10=myosin, Heavy Chain 10, Non-Muscle; Gna11=Guanine Nucleotide Binding Protein, Alpha 11; S61A1=Sec61 Alpha 1 Subunit; CD82=Cluster of Differentiation 82; FCS=Fetal Calf Serum; TSP=Trimethylsilyl Propionate; ppm=Part Per Million; HPLC=High Performance Liquid Chromatography; MALDI TOF=Matrix Assisted Laser Desorption Ionization Time of Flight; HCCA=α-Cyano-4-Hydroxycinnamic Acid; TFA=Trifluoroacetic Acid; PMSF=Phenylmethanesulfonyl Fluoride; DTT= Dithiothreitol; rpm=Revolutions per minute; G6PD=Glucose-6-Phosphate Dehydrogenase; NADP+=Nicotinamide Adenine Dinucleotide Phosphate; NAD+=Nicotinamide Adenine Dinucleotide; LDH= Lactate Dehydrogenase; S.D.=Standard Deviation; nmMHC=Nonmuscle Myosin Heavy Chain; nmMHC-A=Nonmuscle Myosin Heavy Chain A; nmMHC-B=Nonmuscle Myosin Heavy Chain B; ATP=Adenosine 5'-Triposphate.

Introduction

Chinese hamster ovary fibroblasts (CHO–K1) cells are a good model to study the transformation of microtubules and nuclear DNA during cell cycle. The standard CHO-K1 cell displays the classical stigmata of malignantly transformed cells grown *in vitro* [1]; it has been reported that adenosine 3',5' cyclic monophosphate (cAMP) causes cell differentiation [2] and in particular it causes CHO-K1 cells to lose these transformation characteristics and to assume the morphological habitus approaching that of normal fibroblasts. This process is named "reverse transformation" (RT) [3].

It was found that untreated and cAMP treated cells differ only in terms of the three-dimensional organisation and orientation of microtubules and are quite similar in terms of the amount of polymerised tubulin [3-7]. Microtubules and chromatin-DNA structure have impact on various biological processes such as the control of gene expression, the protein synthesis, the cell cycle regulation and the cell transformation [8-11]. Transformed cells frequently exhibit instability in chromosome number even in clonal populations. Defects in the cytoskeletal structure could well be reflected in corresponding defects in the spindle, which is made of microtubules and which would result in errors of chromosomal distribution among the cell progeny.

However, the changes in the concentration of the different metabolites and the different protein composition associated with morphological changes observed are largely unknown [4]. Puck et al. proposed that the cytoskeleton transmits information from the cell membrane to chromosomal loci in the nucleus and is an important element in regulation of the exposure process [12,13]. The mechanism of the transformation in properties here described is as yet obscure. The phenomena make clear that a fundamental change in properties of the cell membrane has occurred.

We had analyzed the CHO-K1 cell protein composition, in particular of the nuclear compartment, by mass spectrometry (MS) to identify changing in the protein pattern before and after the reverse transformation [14]. We identified three proteins, Myh10, Gna11 and S61A1 present in the nucleus of untreated cells and absent after cAMP exposure. Moreover we identified another protein, Cd82Mouse, which concentration is halved after the differentiation.

The goal of this paper is to investigate the correlations between changing in the cellular metabolism and in the cellular protein expression after RT, in order to obtain more information on this cellular process. To this aim we coupled two powerful techniques: nuclear magnetic resonance (^{1}H-NMR) and MS. We think that this approach could be of wide application in the study of many cellular systems.

We choose ^{1}H-NMR since it is revealed an extremely useful and a noninvasive technique in monitoring cell metabolism [15–18]. Since it is known that CHO–K1 cells show different growth features depending on the growth medium, we first studied the variation in CHO–K1 metabolism with the addition of 10% fetal calf serum (FCS), observing

***Corresponding author:** Claudio Nicolini, President Nanoworld Institute Fondazione EL.B.A.Nicolini, Largo Redaelli 7, Pradalunga, Bergamo, Italy, E-mail: claudio.nicolini@unige.it

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a relevant change in the glucose metabolism and in the features of plasma membrane. We have investigated if these variations depend on the cell cycle phase.

^{1}H-NMR experiment was then performed on samples treated with cAMP, and the variations in terms of glucose and lipid metabolism are analyzed. Moreover, to confirm and support NMR results we performed biochemical assays [19,20] to quantify the amount of the metabolites.

Starting from the results obtained on the metabolism of CHO–K1 grown without and with cAMP we investigated the correlation between the presence of different metabolites and different proteins in cell nucleus.

Materials and Methods

Cell preparation

CHO–K1 fibroblasts were supplied by American Type Culture Collection, Rockville, MD. Cells were cultured at 37°C, under 5% CO_2 humidified atmosphere with F12 medium supplemented with 10% Fetal Calf Serum (FCS), 2 mM glutamine and 100 units/ml of gentamycin. Other samples were cultured in the same medium supplemented with other 10% FCS. The generation time of these cells in these media is approximately 13 hours.

Other cells were treated with a solution 10^{-3} M adenosine 3',5' cyclic monophosphate sodium salt (Sigma Chemical Co., St. Louis, MO) for six hours before the analysis. Normal fibroblasts grow on glass or plastic surfaces but not in suspension, whereas tumoral cells usually grow well in either situation. It has been shown that single cells of CHO–K1 in the native state grow equally well on plastic surfaces or in suspension. In the presence of RT conditions, however, excellent growth is still achieved on the plastic surface but no growth occurs in suspension [3]. We then monitored CHO–K1 reverse transformation monitoring their growing behavior.

Mitotic cells were obtained by colcemide block (0.1 mg/ml for 15 hours), cells in early S phase by hydroxyurea (2 mM for 15 hours), and in G1 phase by Timidin (5 mM for 4 hours) and (after changing F12 medium) hydroxyurea (2 mM for 15 hours).

NMR experiments

For ^{1}H-NMR experiments 2×10^7 cells were collected and dissolved in 1 ml of phosphate-buffered, pH=8 with 10% D_2O. Trimethylsilyl propionate (TSP) was used as internal standard. Five samples of cells, grown each in different conditions, were analyzed and the numeric data we presented here are the average of five experiments.

^{1}H-NMR experiments were recorded, under fully relaxed conditions, on Bruker AMX 500 MHz on a Silicon Graphics SGI Mod. O_2 (O.S. IRIX 6.3).

The probe temperature was maintained at 300 K and water suppression was carried out using the Watergate and the DPFGSE scheme. The spectral width was about 16 ppm. The experiments were acquired with *p1*=9 μs. In order to obtain a total relaxation we collect experiments at different d1 values, starting from *d1*=5 ms to *d1*=30 ms. Each spectrum is the result of 128 scans. The spectra were Fourier transformed and manually phase corrected; an automated baseline was applied.

On each sample a 1D spectrum was acquired every 15 minutes to test the stability of the cell at the analysis temperature. The spectra remained very stable for about 1 hour.

Protein extraction

A commercial protein extraction kit (Subcellular Proteome Extraction Kit, Calbiochem) was chosen for the total protein extraction. For the sequential extraction of the cell content, the kit takes advantage of the different solubility of certain subcellular compartments in special reagent mixtures.

Upon extraction of culture cells, four partial proteomes of the cells were obtained: cytosolic proteins, membrane and membrane/organelle proteins, nucleic proteins, cytoskeleton proteins. The subcellular extraction was performed according to the included protocol for freshly prepared adherent culture cells. We performed extraction from 10^6 CHO–K1 cells in logarithmic phase at 80% confluence. To monitor the extraction procedure, morphological changes of the cells were examined by a phase contrast microscope (Wilovert, Wesco). The protein fractions were stored at –80°C until the MS analysis. The amount of total proteins recovered from each extraction step has been evaluated by Bradford assay [21], using bovine serum albumin as standard.

Mass Spectrometry experiments

The identification of CHO-K1 proteins via mass fingerprint was performed coupling high performance liquid chromatography (HPLC), to separate the proteins, and matrix assisted laser desorption ionization time of flight (MALDI TOF) MS, to analyse the tryptic digest of these samples.

The HPLC measures were carried out on a Varian Star HPLC system which includes: 9012 Gradient Solvent Delivery System, 9050 UV-VIS Detector, 9300 Refrigerated AutoSampler (fitted with a 20 μl loop), and a Star Chromatography Workstation software (version 5.31; Varian Inc., USA). Proteins were separated on a C18 (250×4.6 mm; 5 μm particle size) reverse phase column (Macherey–Nagel, Germany).

The samples eluted from HPLC were digested with trypsin, according to the enzyme supplier (Sigma–Aldrich). For MALDI TOF MS the tryptic digest samples were diluted in a 0.1% Trifluoroacetic acid (TFA) solution and the sample was analyzed as described in [14]. The matrix used was a saturated solution of α-Cyano-4-hydroxycinnamic acid (HCCA) (Bruker Daltonics) dissolved in 2/3 of 0.1% TFA and 1/3 of acetonitrile. 1.5 μl of matrix solution was mixed with 1.5 μl of sample, and then 1 μl of this mixture was spotted onto a suitable aluminum plate and air-dried. MALDI TOF MS was externally calibrated using peptide calibration standard solution (Bruker Daltonics), resulting in a mass accuracy <10 ppm for peptides. The mass lists such obtained were submitted to a data bank search. We used specific software for protein data interpretation, Biotools (Bruker Daltonics), that has a MASCOT Intranet search software (Matrix Sciences, Ltd. www.matrixscience.com) fully integrated allowing an automated protein identification via library search.

Cell extracts preparation

CHO-K1 cells, cultured in the different conditions described, were resuspended in PBS (3.0×10^6 cells/ml) and sonicated once for 10 sec in ice in the presence of 1.0 mM PMSF and 100 mM DTT. Cell debris was removed by centrifugation at 2000 rpm for 20 min. The supernatant was collected and used to detect glycolitic enzyme activity. Proteins were quantified by the Bradford method [19].

Biochemical assays

Biochemical (*enzymatic*) assays, involving the measurement in

absorbance changes, were performed at room temperature (about 25°C) in a Jasco 7800 spectrophotometer.

Glucose-6-phosphate dehydrogenase (G6PD) activity was measured according to Beutler and Fairbanks [22] by monitoring the reduction of $NADP^+$ at 340 nm. The assay mixture contained 0.5 M $MgCl_2$, 20 mM $NADP^+$ and 0.1 M G6P in 100 mM Tris/HCl, pH 8.0. Each reaction kinetic was linear throughout the 10 min of observation. Enzymatic activity was expressed as µmol of NADP-reduced/min/mg of cell protein.

The content of lactate was measured as NADH production, catalyzed by lactic dehydrogenase, using an excess of NAD^+ as substrate [20]. The assay medium contained 240 mM KCl, 20 mM $MgCl_2$, 25 mM NAD, L-LDH 2.5 KU. The reduction of NAD, which is directly proportional to lactate formation, was measured by the increase in 340 nm absorbance. The assay was standardized using a lactate concentration curve. Lactate concentration was expressed as mM/l per 10^6 cells.

Data are the mean ± standard deviation (S.D.) of at least three independent determinations. Statistical analysis was performed using the unpaired Student's *t*-test, considering significant the difference between control and each treatment at least at 5% level ($p<0.05$).

Results

In order to investigate the presence and the concentration of different metabolites, 1D NMR spectra of the CHO-K1 cells were acquired to follow known chemical groups showing distinct resonances, namely:

- α-glutamine and α-glutamate groups at about 3.7 ppm
- inositol at about 3.6 ppm
- choline-containing metabolites (*i.e.*, choline, phosphocoline and glycerophosphocholine) at about 3.2 ppm
- creatine with phosphocreatine at about 2.9 ppm
- glutamate at about 1.8 ppm
- alanine at about 1.4 ppm
- lactate at about 1.3-1.2 ppm
- CH_2 and CH_3 arising from fatty acyl chains of lipids resonating at 1.2-1.1 ppm and 0.8-0.7 ppm

This allows monitoring the variation in the concentration of metabolites of CHO-K1 cells grown in a medium enriched by 10% FCS with respect to cells grown in the control medium. We indeed observed important variations in lactate and choline concentration (Figure 1) up to 25% increase for lactate, revealing an increasing of the glucose metabolism of the cells. In fact, his production is augmented when glycolysis is increased. Also the alanine and glutamate peaks show significant positive variations in treated cells, confirming an activation of cell energy metabolism. Another significant variation is observed in the height of the choline peak, indicating a change in lipid metabolism, since choline, phosphocoline and glycerophosphocholine are three important lipid precursors. The mean concentration of choline is diminished of 42%. The change in the Choline-containing metabolites suggests a change in the plasma membrane and in its characteristics (permeability, fluidity, etc.). In figure 3 we report the mean changes and the standard deviation on five experiments.

Then we have checked that these variations are not dependent on differences in the synchronization of cells in the examined samples. The same experiments were repeated on cell samples blocked in different phases of the cell cycle (M, G1 and early-S), in order to verify that ^{1}H-NMR variations are not dependent upon differences in cell proliferation. Three samples of CHO–K1 grown in control medium and three samples of cells grown in 10% FCS were blocked respectively in

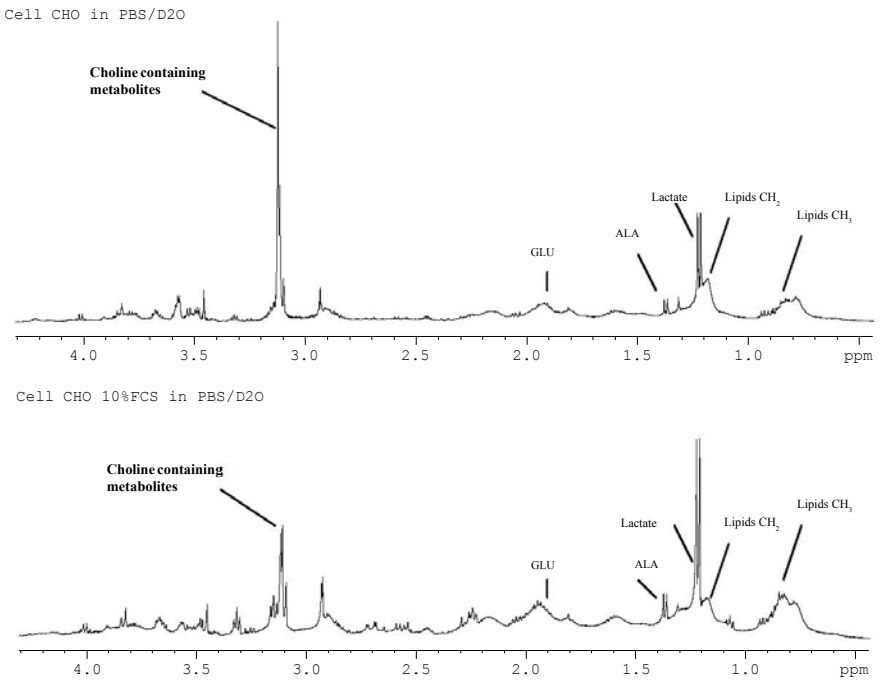

Figure 1: 1D NMR spectrum of CHO–K1 cells grown in FCS enriched medium. Expansion 0.5-4.5 ppm of 1D NMR spectrum of CHO–K1 cells grown in normal medium (upper) grown in medium enriched by 10% FCS (lower).

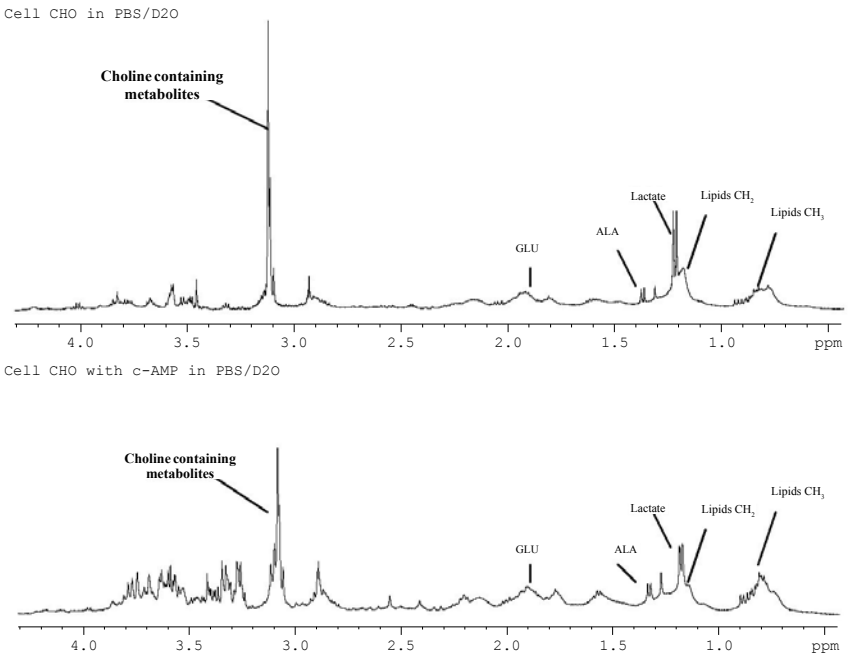

Figure 2: 1D NMR spectrum of CHO–K1 cells grown with cAMP. Expansion 0.5–4.5 ppm of 1D NMR spectrum of CHO–K1 cells grown in normal medium (upper) and grown with cAMP (lower).

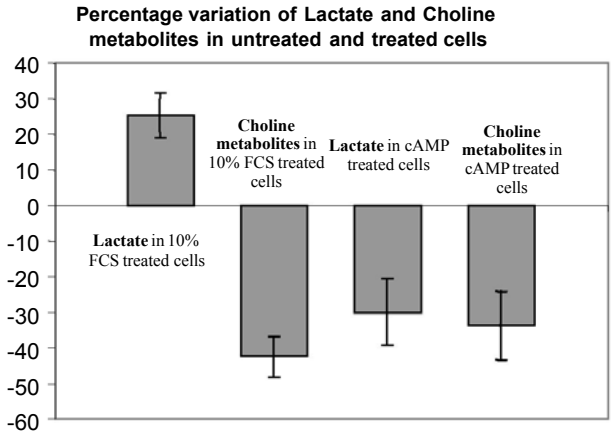

Figure 3: Metabolites Percentage variations. Percentage variations of lactate and choline-containing metabolites in FCS and cAMP treated cells towards control.

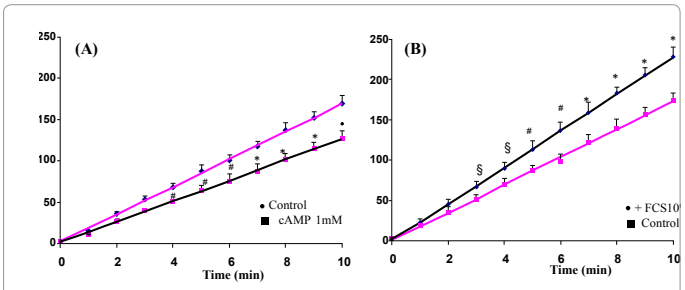

Figure 4: G6PD activity. Time-course of G6PD activity: G6PD activity (U/mg) was measured in CHO–K1 treated with 1mM cAMP for 6 h (A) and grown in a medium supplemented with 10% FCS (B). Data are the mean ± S.D. of three experiments. *p<0.0125, #p<0.025, p<0.0125; §p<0.05 vs. control.

M, G1 and early S phase of cell cycle. NMR 1D spectra of synchronized M, G1 and early S CHO–K1 show overlapping peaks and have the same intensities respect to the cell control sample (logarithmic phase) grown in the same medium (data not shown). This result is in agreement with the data observed in the literature [17]. Consequently one can conclude that it is not the cell cycle progression to induce the observed change in cell metabolism.

We also analysed the cAMP treated cells (Figure 2). Comparing the NMR spectra with the ones obtained from cells in control medium, we observed again differences in choline containing metabolites, lipids and lactate concentration.

From the comparison between the control and cAMP treated cells spectra, we can observe that also in this case the glucose and lipid metabolism are influenced. For the peaks of Choline-containing metabolites, lipid CH_2 and lipid CH_3 a decrease of 30% (mean value) is observed. Also the concentration of Lactate is decreased of about 33%, suggesting a change in bioenergetics and the metabolic pathway of cells.

To confirm the alterations suggested by NMR analysis G6PD and lactate formation were then biochemically assayed. G6PD is indeed the key enzyme for regulation of intracellular redox level during cell growth [23]. In CHO–K1 cells G6PD activity, after 10 min, corresponds to 170 U/mg. The exposure of CHO-K1 cells to 1 mM cAMP reduces of about 26% enzyme activity. The effect is already significant after 4 min ($p<0.05$) (Figure 4A). Moreover, as shown in figure 4B, in CHO–K1 cells grown in the medium enriched by 10% FCS, the G6PD activity increases of about 24% respect to cells grown in the control medium.

The lactate levels were measured in CHO–K1 cells pre-incubated

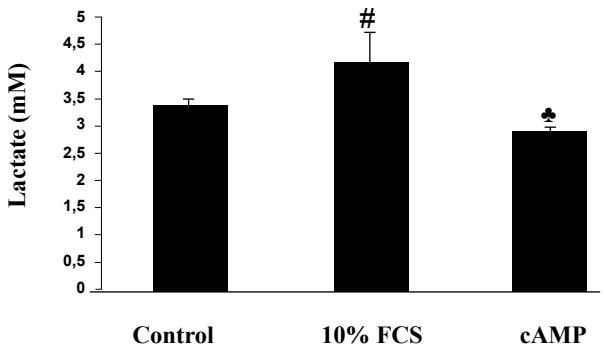

Figure 5: Effect of cAMP and FCS on Lactate formation. Lactate levels (mM) were measured in CHO-K1 treated with 1mM cAMP for 6 h and grown in a medium supplemented with 10% FCS. Data are the mean values ± S.D. of three experiments, #p<0.025, ♣p<0.01 vs. control.

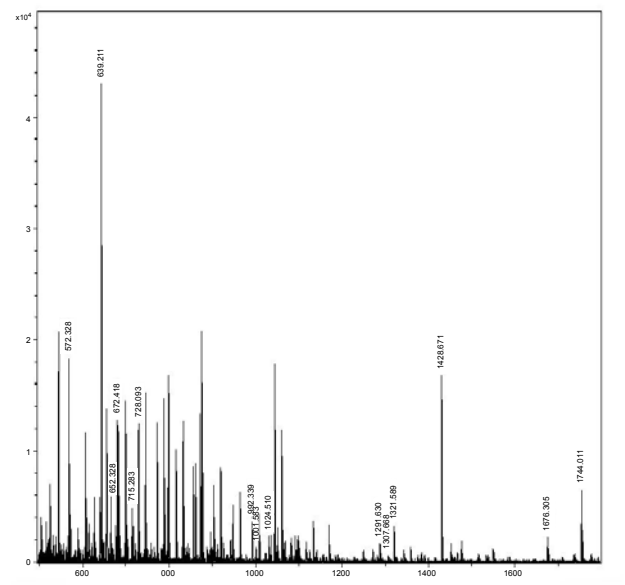

Figure 6: Mass spectrum of Myh10 tryptic digest. The mass list obtained allowed the identification of Myh10 by Mascot databank search. The Mascot Search Results are reported in the windows.

with cAMP and FCS. The results, shown in figure 5, indicate that cAMP induced a reduction of about 24% of lactate formation; moreover, in presence of FCS the levels increased about 18%. Comfortingly these data appear in strict correlation with NMR results.

Finally we have investigated the same CHO–K1 cells, untreated and cAMP treated, with MALDI TOF MS and we have identified, by peptide fingerprint, the key nuclear proteins which better correlate with the cAMP induced cell differentiation (Figure 6).

Discussion

CHO–K1 cells metabolism is probed by ^{1}H–NMR and biochemical assays in different experimental conditions, namely growth in normal medium, in medium enriched by 10% FCS and after reverse transformation by cAMP. In order to obtain complementary informations and to study different aspects of cells we related these results with those independently obtained by MS analysis of nuclear proteins [14].

We started from the data known in literature [1-4,12,13] and from the results obtained also in our laboratory some years ago [5,21] indicating that CHO-K1 cells are sensitive to the presence of FCS in growth medium and that in presence of cAMP the shape of cells changes from polygonal to elongated.

First of all, we tested NMR technique in the studying of cellular metabolism; comparing the ^{1}H-1D spectra of control cells with the spectra of 10% FCS-treated cells. As expectable we found an increase in cell energy metabolism, revealed by an increased concentration of lactate (involved in glycolisis process). Also a change in membrane lipid concentration was observed, that lead to hypothesize some conformational changes in the membranes of these "activated" cells. Further analyses are in progress to verify this hypothesis. On the other hand, we have analyzed the cells treated with cAMP and we have compared the obtained spectra with the untreated ones. Also in this case we have observed a decrease in choline containing metabolites and in the concentration of lipids, indicated from the decreased intensity of peaks arising from methyl and methylene groups of fatty acyl chains of lipids. This change in lipid concentration may correlate with the structural changes occurring to cells when they were treated with cAMP.

To support the results obtained by NMR analysis we performed biochemical assays. We measured lactate and G6PD formation, by measuring the absorbance changes, to have a bioanalytical determination of the levels of lactate and to confirm that the lactate levels were directly related to the rates of glycolisis of the cell. The data obtained confirm NMR results.

We investigated the same CHO-K1 cells, untreated and cAMP treated, with Mass Spectrometry [14]. Our MS results showed different protein contents in the nuclear protein fraction of the two samples of cells. In particular we identified three proteins, Myh10, Gna11 and S61A1, present only in the nucleus of untreated cells and involved in cellular regulation processes.

Led by NMR results we analyzed the function of the gene corresponding to the expressed protein analyzing the correlation between this function and the observed changes in metabolites.

Within the myosin family of proteins, the conventional myosin II isoform falls into two subgroups: the sarcomeric (skeletal and cardiac muscles) and the nonsarcomeric (smooth muscle and nonmuscle). There are evidences that nonmuscle myosins play a role in diverse cellular functions, for instance cytokinesis, proliferation, secretion, and receptor capping. Myosin molecule consists of a pair of heavy chains and two pairs of light chains. Two isoforms of the nonmuscle myosin heavy chain (nmMHC), chain A (nmMHC-A) and chain B (nmMHC-B) have been identified. The nmMHC-B in particular is involved in cell growth regulation and transformation [24]. The Myh10 protein is involved in the catalysis of movement along a polymeric molecule such as a microfilament or microtubule, coupled to the hydrolysis of adenosine 5'-triposphate (ATP). The processes regulated by this protein require the presence of energy and it was found to be absent in cAMP treated sample, when the metabolism of glucose was decreased, confirming a lower energetic need of the cell.

Heterotrimeric guanine nucleotide-binding proteins (G proteins) are integral to the signal transduction pathways that mediate the response of the cell to many hormones, neuromodulators, and a variety

of other ligands [25]. The Gna11 protein regulates the cascade of processes by which a signal interacts with a receptor, causing a change in the level or activity of a second messenger and effecting a change in the functioning of the cell. Also this protein is absent in cAMP treated sample, when the membrane features are strongly changed meaning that also the introduction of the signal molecules into the cell can be modified.

The change in lipid concentration and consequently in cellular membranes can be correlated also with the expression of S61A1 protein since it regulates the process of targeting specific proteins to particular membrane-bound subcellular organelles.

Moreover we identified another protein present in both samples but in a double concentration in the nucleus of untreated cells, Cd82 Mouse that associates with CD4 or CD8 delivers co stimulatory signals for the TCR/CD3 pathway [26] involved in apoptosis regulation.

These results on the nucleus proteins encouraged us to perform the same analysis on the proteins of other cellular compartments (cytosol, membrane and membrane/organelle and cytoskeleton) to analyse how the whole protein pattern of CHO-K1 cells changes after RT and how these changes are correlated with the metabolism alterations here revealed. These results will give us a better understanding of the correlation between cellular morphologic/metabolic alterations and protein expression pattern.

In conclusion the combined utilization of ^{1}H-NMR, biochemical assays and Mass spectrometry shed new light in our understanding of cell metabolism and protein composition during reverse transformation. Other studies will be necessary and are in progress to better understand the complexity of such processes that regulate cell metabolism and protein expression.

Acknowledgments

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