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Differentiation-related changes in myogenic stem cells

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Skeletal myoblast cells have a high potential for cell therapy based repair of the injured myocardium. Stem cell differentiation markers are important for finding an appropriate cellular state optimal for engraftment. We followed differentiation-induced changes in cellular size, the distribution of mitochondria, adenine and pyridine nucleotide content, NAD(P)H fluorescence in skeletal myogenic stem cell populations derived from rabbit skeletal muscle. The fraction of larger size cells increased during differentiation. Mitochondrial distribution changed from perinuclear in undifferentiated cells to even in the cytoplasm of differentiated cells. The most remarkable feature of undifferentiated myoblasts was a very low NAD(P)H fluorescence which augmented after initiating cell differentiation, due to metabolic and physiological changes, but that did not correlate with an increase in cellular size. A significant increase of NAD(P)H concentration was chromatographically detected after 8 days of differentiation, although the NAD(P)H fluorescence increased by 42% as soon as after three days and 2.7-fold after five days of differentiation as compared with the control. We show that the early increase in fluorescence is determined by mitochondrial NADH, but upon a longer differentiation NADPH amount also increases. The total amount of adenine nucleotides (ATP, ADP, AMP) increased, but the ATP / ADP ratio decreased during differentiation. We suggest that an increase in NAD(P)H fluorescence may be useful for a non-invasive detection of the onset and course of myoblast differentiation.

Key words: myogenic stem cells, differentiation, NAD(P)H fluorescence

INTRODUCTION

The high therapeutical potential of skeletal myoblasts makes important a thorough understanding of cell differentiation process. The changes stem cells undergo during differentiation include transformations of cell shape, ultrastructure, biochemical composition and metabolism. The goal to employ cells for regenerative medicine implies the necessity of knowing how to track and control the differentiation process. Noninvasive and rapid methods to monitor the metabolic and viability state in differentiating cells are under extreme request. The pyridine dinucleotides NAD^+ / NADH and $NADP^+$ / NADPH are important indicators of cell viability and energy metabolism state. Taking into account that their reduced forms are natural cellular fluorophores, it is reasonable to verify how NAD(P)H fluorescence correlates with numerous changes in cell state during differentiation. This should help to estimate to what extent NAD(P)H fluorescence measurements may be useful for a noninvasive analysis.

When a cell enters the differentiation process, it switches the metabolism from anaerobic to aerobic [1]. The expression of mitochondrial enzymes and the respiration rate increase and the mitochondrial distribution changes from perinuclear to even in the cell volume [2]. Although several authors have focused on changes in adenine nucleotides [1, 2], the content of pyridine dinucleotides in stem cells and their changes during differentiation have not yet been characterized.

The aim of this study was to estimate NAD(P)H fluorescence in parallel with changes of the cellular distribution of mitochondria, the size of cells, changes of adenine and pyridine nucleotide concentration in skeletal myogenic stem cells during differentiation.

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METHODS

Cell cultivation and differentiation. Experiments were performed on the rabbit myogenic stem cell line Fr3, derived from rabbit skeletal (femur quadriceps) muscle, a the Institute of Cardiology, Laboratory of Cell Culture, Kaunas University of Medicine (Kaunas). Cells were cultivated in Iskove's modified Eagle medium (IMEM) supplemented with stable L-glutamine (2 mM), foetal calf serum (10%), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Biochrom AG, Germany). Cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2 and passaged twice a week detaching cells from the substrate with 0.25% (w/v) trypsin solution (Biochrom AG, Germany). Cell differentiation was induced by culturing 80% confluent cells in IMEM supplemented with 2% horse serum. Cells were cultivated in 75 cm^2 flacons; the cultivation medium was changed 24 h before the experiment. Experiments were performed with cells on the 3rd, 5th, 8th, 10th and 12th days of differentiation. The differentiation process was confirmed by immunofluorescent detection of a myogenin-specific differentiation marker in muscle cells. Primary mouse monoclonal antibodies to myogenin with secondary Chromeo488 conjugated goat polyclonal antibodies to mouse IgG (Abcam, UK) were used (data not shown).

NAD(P)H fluorescence measurement. A cell monolayer was washed with PBS twice. The cells were detached from the substrate by 0.25% (w/v) trypsin solution, resuspended in 10 volumes of PBS and centrifuged for 10 min at 1000x g. The pellet was resuspended in PBS and centrifuged as previously described. Cells were resuspended in PBS to a final concentration of $5 \cdot 10^6$ cells/ml. The total cell number and viability were evaluated using a trypan blue test. The total fluorescence of NADH and NADPH was measured in 75 μl of suspension in black 96 well plate using a Tecan GENios Pro™ fluorimeter and XFluor™ software (Tecan Group Ltd., Switzerland). The excitation wavelength was 340 ± 10 nm and the emission wavelength was 460 ± 10 nm. PBS buffer fluorescence was used as blank.

The minimum and maximum NAD(P)H fluorescence values were determined using non-fluorescent oxidative metabolic state effectors: 0.1 mM carbonyl cyanide 3-chlorophenylhydrazone (CCCP, uncoupler of mitochondrial oxidative phosphorylation) was used to obtain the minimal NAD(P)H fluorescence, and 3 mM KCN (inhibitor of cytochrome oxidase) was used to obtain the maximal NAD(P)H fluorescence. Previously, these methods had been used to study the effects on the NADH signal by Eng et al. [3].

Microscopic analysis. Cell morphology and MitoTracker Green fluorescence were imaged with a Motic BA400 fluorescence microscope combined with a digital high-resolution Moticam 5 000C camera. Motic Images Advanced 3.2 software (Motic, Germany) was used to calculate cell size area (μm²). A 40× objective was used for light microscopy, and 100× immersive objective (Biometrija, Lithuania) and thermostatic microscope board with controller (Biometrija, Lithuania) were used for fluorescence microscopy. Stained cells were imaged with the green fluorescence channel ($\lambda_{excitation}$: 450–490 nm band pass filter, $\lambda_{emission}$: >515 nm long pass filter). The illumination time required for optimal fluorescence image acquisition was manually set and varied within 300–500 ms.

Cell staining with MitoTracker Green FM dye. Cells were seeded in 4 cm diameter Petri dishes (500 cells per dish) for microscopic assay. The stock solution of MitoTracker Green FM (Molecular Probes Invitrogen GmbH, Germany) was prepared by dissolving 50 μg of dye in 250 μl DMSO (Carl Roth, Germany) to the final concentration of 300 μM. Cells grown in Petri dishes were washed three times with 1 ml IMEM without serum; 10 μl of the stock solution and 990 μl of a serum-free medium were applied onto the cells to achieve the final dye concentration of 3 μM. Cells were incubated for 30 min at 37 °C in a CO_2 incubator in the dark. The incubated cells were washed three times with 1 ml of a serum-free medium, and 2 ml of the medium was added prior to microscopy.

HPLC analysis. Cells were seeded in 9.6 cm diameter Petri dishes ($2.5 \cdot 10^5$ cells per dish) for the chromatographic analysis of adenine and pyridine nucleotide concentration. A cell monolayer was washed three times with PBS and disrupted with Drygalski spatula in 1 ml of ice-cold 0.5 M KOH. Samples were incubated on ice for 3 min, then 840 μl of a sample was mixed with 170 μl of 8.5% H_3PO_4 and incubated on ice for 3 min. After incubation, the extract was cleared by a 3-min centrifugation at 1000× g, 4 °C. The supernatant was filtered through a syringe filter with a 0.45 μm pore diameter PVDF membrane. The prepared samples were kept at –80 °C and analyzed within 48 hours. HPLC analysis was performed using an Agilent 1 200 Series system consisting of a diode array detector, a manual injector with a 50 μl loop, a vacuum degasser, a quaternary pump, a Symmetry 3.5 μm, 4.6 × 100 mM column with a precolumn (Waters, Ireland) and ChemStation 3D LC software for data-processing (Agilent Technologies, Germany). We used a modified analysis procedure [4]: column temperature 25 °C, injection volume 50 μl. Eluents – A: 10 mM KPi pH 5, 2 mM TBA (tetrabutylammonium bromide), 3% acetonitrile, and B: 10 mM KPi pH 7.5, 2 mM TBA, 30% acetonitrile. Gradient elution used: B 0 min – 1%, 30 min – 95%, 32 min – 1%. For detecting adenine and pyridine nucleotides, 259 nm and 340 nm absorption wavelengths were used.

RESULTS AND DISCUSSION

When myoblasts differentiate into myotubes, the cells undergo a profound reorganization [5–7]. The differentiation induced in rabbit myogenic stem cells was monitored in a

trypsinized Fr3 stem cell suspension by light microscopy, registering changes in size determined as 2D cell projection in micrographs, the shape and intracellular structure of the cell (Fig. 1). The dynamics of cell size distribution during differentiation and their mean size are presented in Fig. 2. Undifferentiated cells were small and round (Fig. 1). The mean size of the cell in the population was 109 μm^2, but two populations could be distinguished: the surface area of 80% of cells was 50–150 μm^2 and of 20% 150–250 μm^2.

Cells increased in size and changed their shape in the course of differentiation. After three days of differentiation, myoblasts significantly increased in size (mean size

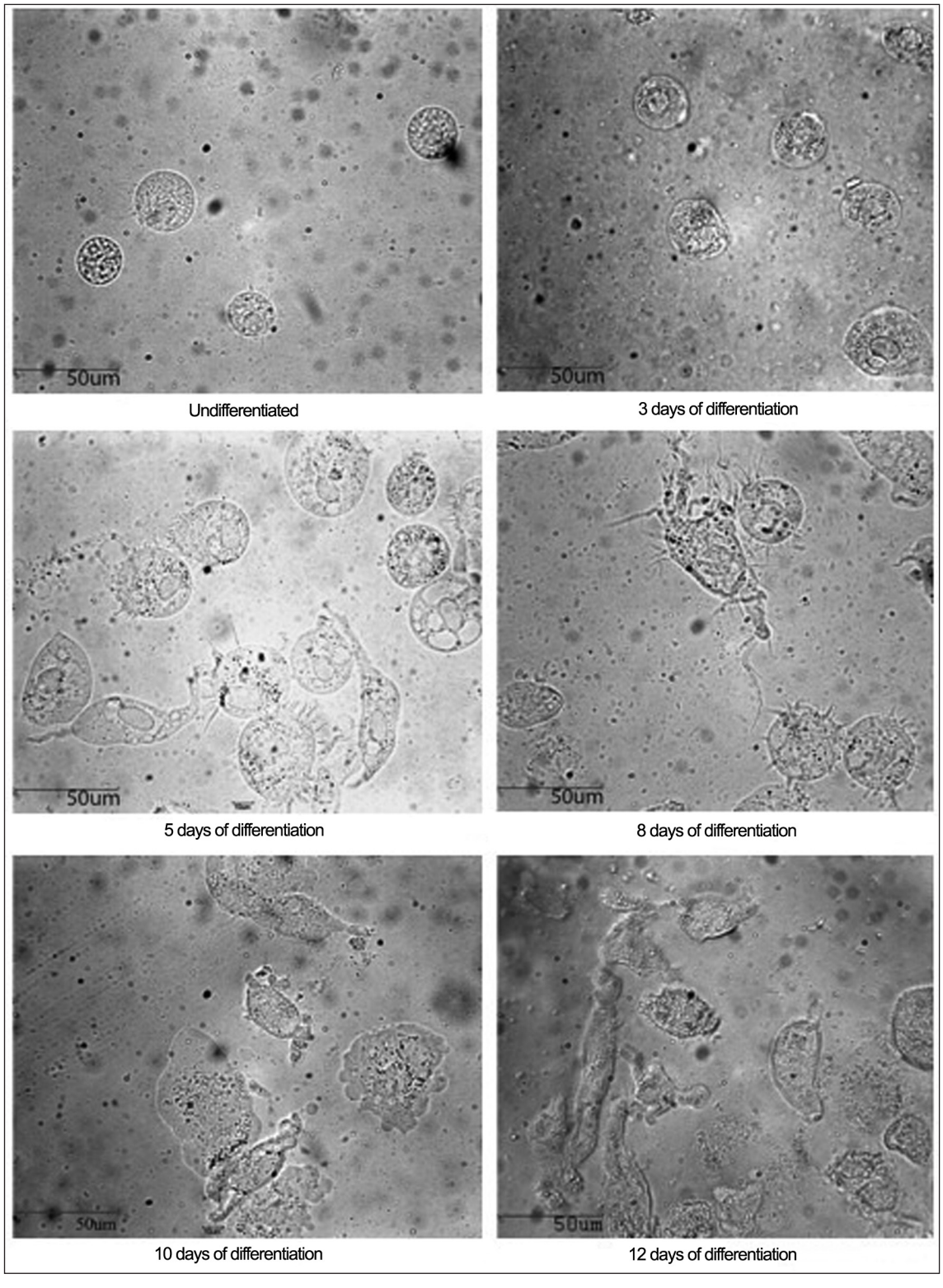

Fig. 1. Morphological changes of Fr3 line cells during differentiation

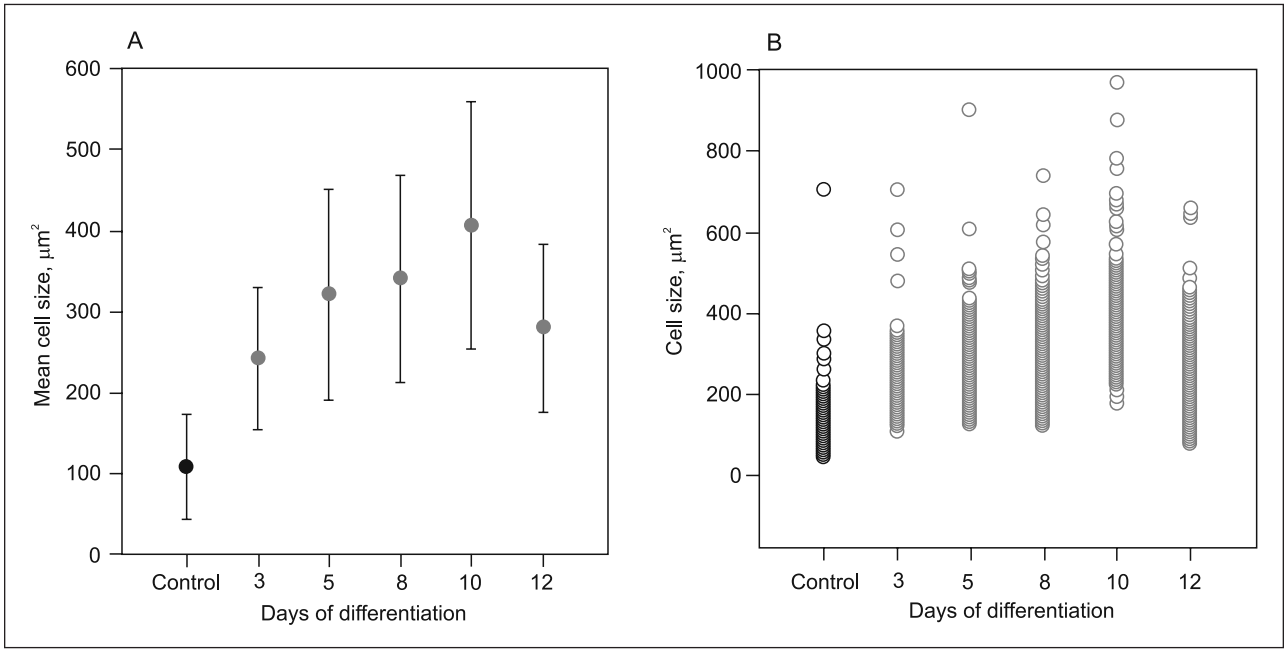

Fig. 2. Changes in the size of Fr3 line cells during differentiation: A – the mean of size (average from n = 3 independent experiments ± SD); B – distribution of the cells in the population by cell size

242 μm²) compared to the control (Figs. 1 and 2). The population was comprised of several cell fractions: 10% – 100–150 μm², 56% – 150–250 μm² and 34% – 250–350 μm².

Clear morphological changes were observed after five days of differentiation: cells continued enlarging intensively (mean size 321 μm²), and the cell size heterogeneity in population increased: the biggest cells reached 450–550 μm² and comprised 7% of the population; smaller cells made three main fractions of 350–450 μm² (20%), 250–350 μm² (39%) and 150–250 μm² (25%); the remaining 6% belonged to the 100–150 μm² fraction. The cells after five days of differentiation were round and spindle-shaped, with abundance of oblong-shaped nucleis; different structures, blisters, inclusions were seen in the cytoplasm; there were excrescences (possible microtubes) on the surface of some cells. After eight days of differentiation the surface excrescences still remained, and the granular inner cell structure could be clearly seen. The cells were slightly enlarged as compared with day 5 (the mean cell size in the population was 340 μm²). A large cell fraction appeared in the population with cell size 550–650 μm² (5%). The distribution of the rest of the population changed negligibly as compared with the distribution by size after five days of differentiation (450–550 μm² – 13%, 350–450 μm² – 21%, 250–350 μm² – 34%, 150–250 μm² – 22% and 100–150 μm² – 5%). The largest myoblasts were observed on the 10th day of differentiation when the mean size of the population was 406 μm² (250–450 μm² cells made 62% and 600–800 μm² cells 14%). After 12 days of differentiation, Fr3 myoblasts became oblong-shaped and started reducing in size (150–300 μm² cells made 50% of population); their viability also slightly reduced.

Recently, some authors have suggested a hypothesis that stem cell competence (i. e. stem cell stability and pluripotency) may be estimated using functional mitochondrial characteristics [8]. Lonergan et al. [2] found that several mitochondrial properties, such as subcellular localization and metabolic activity, can be considered as markers of "stemness". For a better understanding of intracellular changes that stem cells undergo, we evaluated mitochondrial distribution in undifferentiated myogenic stem cells and in cells differentiated for 7 days by staining cells with a MitoTracker Green FM fluorescence probe and monitoring the mitochondria by fluorescence microscopy (Fig. 3). In undifferentiated Fr3 cells, mitochondria were localized in the perinuclear space with some randomly distributed mitochondria in the cytoplasm. There were no multinuclear cells and cells with a well-defined stained intranuclear structure (Fig. 3A). In differentiated Fr3 stem cells, mitochondria were evenly scattered or formed a network in the cytoplasm (Fig. 3B) without a noticeable perinuclear localization. The cells were mononuclear with some rare intranuclear structures.

It had been determined by other authors [1, 9] that morphological changes during differentiation were initiated as a cell differentiation metabolism switched from anaerobic (glycolysis) to aerobic (oxidative phosphorylation), and the nucleus–mitochondria intercommunication was required to translate the signals that regulate cell fate. The cellular NADH fluorescence had been approved long ago as a useful

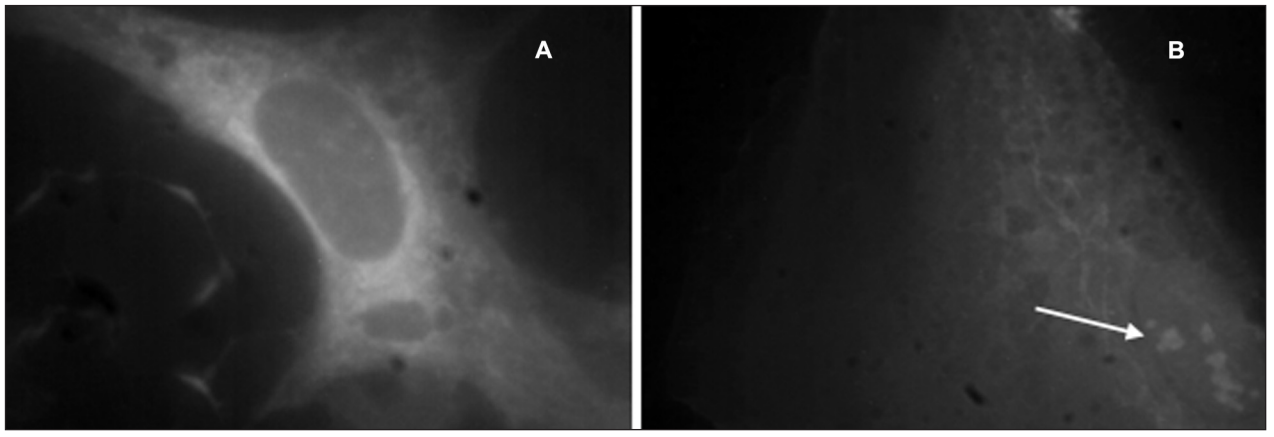

Fig. 3. Mitochondrial distribution in Fr3 stem cells: A – undiferentiated cell, B – differentiated cell (7 days). The arrow points to the nucleus of differentiated cell (mitochondria stained with MitoTracker Green, 100×)

tool to study the mitochondrial function in energy-linked processes and as an optimal indicator of intracellular redox state [10, 11]. Nicotinamide adenine dinucleotides (NAD^+ / NADH and $NADP^+$ / NADPH) are important coenzymes involved in the metabolism of living cells, and their reduced forms (denoted here as NAD(P)H) are endogenous fluorophores. Although NADH and NADPH are distributed throughout the cytosol, studies have shown that the predominant fluorescence signal originates from the mitochondrial NADH [3, 12, 13]. There is no information available about NAD(P)H distribution patterns in myogenic stem cell compartments; therefore, we aimed to estimate differentiation-induced NAD(P)H fluorescence changes and to detect to what extent this fluorescence is attributable to the mitochondrial NADH pool. The data on changes in NAD(P)H fluorescence during differentiation are presented in Fig. 4.

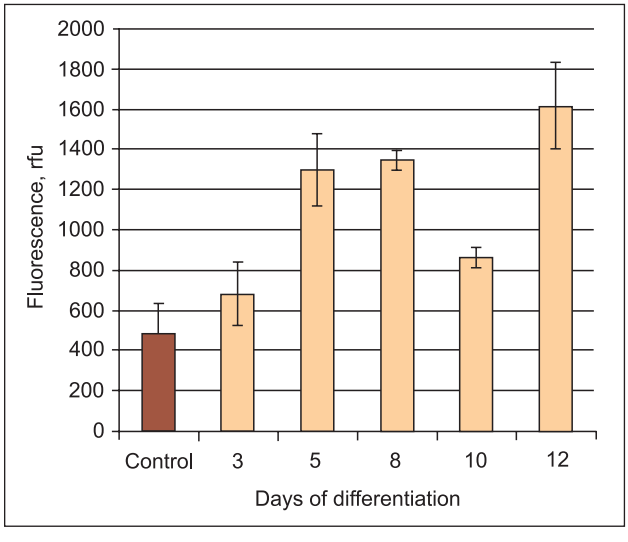

Fig. 4. NAD(P)H fluorescence of Fr3 cells suspension during differentiation (average from four independent experiments ± SD). Fluorescence was registered at excitation λ = 340 nm; emission λ = 460 nm

One can see that undifferentiated stem cells had a very low fluorescence (478 rfu) which increased in the course of differentiation. In three days after the onset differentiation, NAD(P)H fluorescence increased by 42% and in 5 days 2.7-fold as compared with the control. The further three days of differentiation did not change the fluorescence. A correlation between changes in cell size and NAD(P)H fluorescence of the analyzed cells can be estimated by comparing data in Fig. 2 and Fig. 4. One may see that on the 10th day of differentiation the cell size of the population was the largest; however, the fluorescence decreased by 36% as compared to that on the 8th day of differentiation. The cell size of population, on the contrary, tended to decrease after 12 days of differentiation, but the fluorescence increased by 87% as compared with that on the 10th day and by 19% as compared with the 8th day, and was the highest fluorescence achieved in cell suspension. The results show that cell fluorescence does not correlate with cell size but is most possibly determined by the metabolic changes.

It should be noted that the NAD(P)H fluorescence signal depends on the absorption and scattering which result from the nonfluorescent absorbers and scatterers, and also on the fluorescence of other cellular fluorophores having spectra overlapping with those of NAD(P)H excitation and emission [14, 15]. Therefore, we have determined how the measured fluorescence signal is changed by various nonfluorescent chemical agents that modulate the amount of mitochondrial NADH: carbonyl cyanide 3-chlorophenylhydrazone (CCCP), an uncoupler of mitochondrial oxidative phosphorylation, which stimulates NADH oxidation and therefore helps to obtain minimal NADH fluorescence, and KCN, an inhibitor of cytochrome oxidase that blocks NADH oxidation and helps to obtain the maximal NADH fluorescence (Fig. 5). Previously, these methods had been used to study the effects on the NADH fluorescence signal by Eng et al. [3].

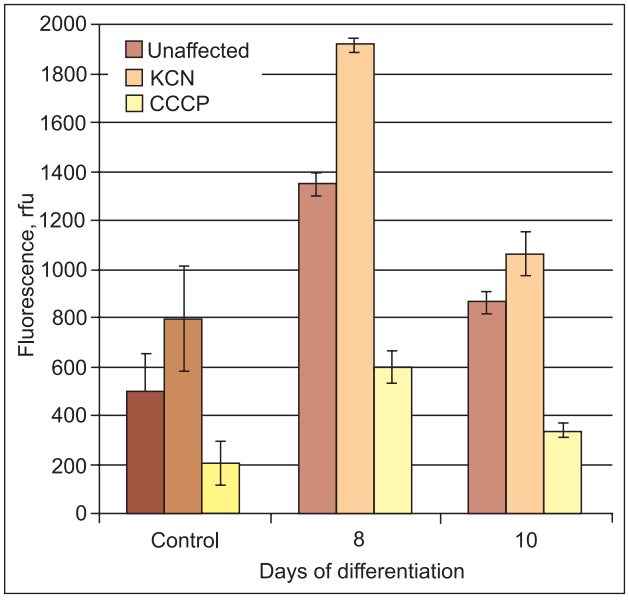

Fig. 5. Effect of KCN and CCCP on NAD(P)H-specific fluorescence of Fr3 cell suspension fluorescence (average from three independent experiments ± SD)

Although CCCP decreased NAD(P)H fluorescence in undifferentiated and differentiated cells to 40% of the fluorescence level of unaffected cells, in differentiated cells the fluorescence after treatment with CCCP was 2.9- and 1.6-fold higher on days 8 and 10, respectively, as compared with control cells. It shows that during differentiation the contribution of other fluorophores (possibly to some extent also of NADPH) tends to increase, but the mitochondrial NADH content also significantly increases. The uncoupling effect (difference in NAD(P)H fluorescence of untreated cells and those affected with CCCP) in differentiated cells was 2.5- and 1.8-fold higher after 8 and 10 days of differentiation as compared with the effect in the control cells. These results show that NADH content in differentiated cells is higher. Inhibition of cytochrome oxidase by KCN caused a increase in NADH fluorescence in dependence on cell differentiation stage. In undifferentiated cells that exhibit an unusually low NAD(P)H fluorescence, KCN increased NAD(P)H fluorescence by 60%, indicating a substantial contribution of mitochondrial NADH to the overall weak fluorescence signal. This increase in differentiated cells was significantly smaller (by 42% and 23%, respectively, for days 8 and 10 of differentiation) since the fluorescence of unaffected cells was much higher. Differences between maximal (KCN-treated) and minimal (CCCP-treated) NAD(P)H fluorescence values (Fig. 5) reflect the relative size of the mitochondrial NAD^+ + NADH pool in different cell populations. One may note that in the control and after 8 days of differentiation this difference is almost equal to the initial fluorescence value, whereas after 10 days of differentiation it is considerably smaller than the fluorescence in unaffected cells, possibly indicating a larger input of some other fluorophores except mitochondrial NADH. However, it is clear from the large effect of CCCP (Fig. 5) that in all cases mitochondrial NADH determines the major part of NAD(P)H fluorescence in cell suspensions.

Aiming to determine the absolute $NAD(P)^+$ / NAD(P)H concentration and the concentrations of interlinked adenine nucleotides (AMP, ADP, ATP) in stem cells during differentiation, we analyzed the cellular extracts by the reverse phase ion-pair high performance liquid chromatography (RP-IP HPLC). Chromatographically determined concentrations of pyridine and adenine nucleotides in Fr3 cell extracts from undifferentiated (control) cells and from the cells after 3, 5, 8, 10 and 12 days of differentiation are presented in Fig. 6. The calculated total amounts of nucleotides, as well as their important ratios are shown in Table.

The results of chromatographic analysis confirmed data of fluorescence measurements indicating that the total amount of the reduced pyridine nucleotides NAD(P)H was unusually low in undifferentiated myoblasts as compared with differentiated cells. However, a significant increase of NAD(P)H concentration was chromatographically detected after 8 days of differentiation, although the NAD(P)H fluorescence increased by 42% as soon as after three days and 2.7-fold after five days of differentiation versus the control. Thus, in addition to the well-known advantages of being non-invasive and very rapid, NAD(P)H fluorescence measurements have appeared to be a highly sensitive method for estimating not only cellular energy status, but also the onset and dynamics of differentiation, and very probably a marker of loosing stemness (if confirmed for

Table. **Total concentrations of adenine and pyridine nucleotides and their ratios**

Days of differentiation	AMP + ADP + ATP, Nmol / 10^6 cells	ATP / ADP	$NAD(P)^+$ + NAD(P)H, nmol / 10^6 cells	NAD(P)H, nmol / 10^6 cells	NADH / NAD^+
Control	6.96	7.00	4.26	0.55	0.10
3	6.65	7.16	4.57	0.33	0.09
5	8.74*	7.93	4.39	0.35	0.10
8	9.76*	6.11*	4.17	0.71*	0.23*
10	13.89*	4.70*	3.96	0.67*	0.16
12	15.12*	5.68*	5.11*	1.31*	0.22*

* Statistically significant difference between control and test groups ($p < 0.05$).

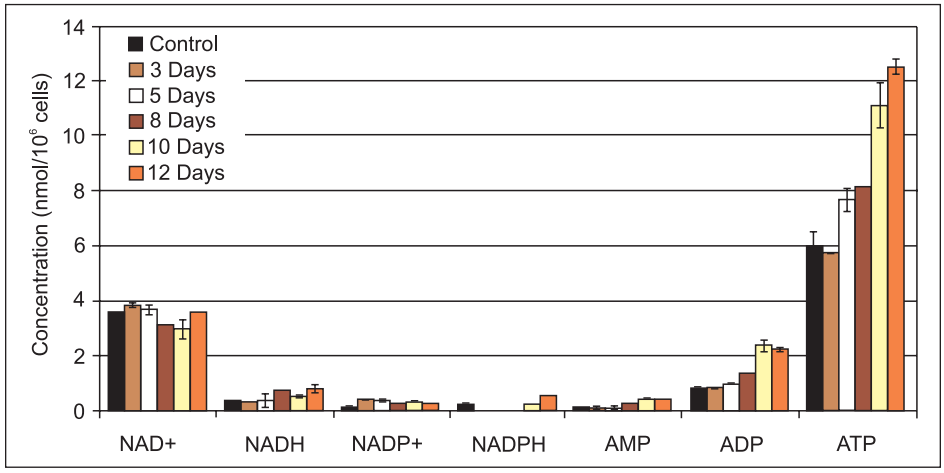

Fig 6. Changes in the concentration of adenine and pyridine nucleotide concentrations in FR3 line cells during differentiation (n = 3, average ± SE)

other stem cell types). The significant changes in NADH concentration and NADH / NAD^+ ratio after 8 days of differentiation are probably related to reorganization of the mitochondria (Fig. 3) that contain the main cellular pool of NADH, and to changes of their functions. The results of NAD(P)H fluorescence and the chromatographically determined concentration correlate well in cells that differentiated longer (Figs. 4 and 6): both values decreased on day 10 and again increased on day 12. The NADH / NAD^+ ratio significantly increased after 8 and 12 days of differentiation. The discrepancy between fluorescence and NAD(P)H concentration on 3rd and 5th days may result from different optic characteristics of bound and free NADH [16]. So far, there are data available on changes of the ratio of bound and free NADH during myogenic stem cell differentiation and a possible impact of these changes on the fluorescence signal.

The total content of oxidized and reduced pyridine dinucleotides ($NAD(P)^+$ + NAD(P)H) did not change significantly with differentiation and increased by 20% as compared with control only after 12 days of differentiation. It is clear that independently of the differentiation stage, the main constituent of the $NAD(P)^+$ pool is oxidized NAD^+ (Fig. 6), indicating that the redox balance in cultured skeletal myoblasts is strongly shifted towards the oxidized state. The amount of both fluorescent reduced forms (NADH and NADPH) increased in essential agreement with the fluorescence measurements, leading to a doubling of the NADH / NAD^+ ratio and a substantial rise in NAD(P)H amount upon a longer differentiation. The latter result is in accordance with the analysis presented in Fig. 5, since it reveals that due to NADPH accumulation the effect of CCCP is smaller upon a longer differentiation.

The activation of cellular energy metabolism upon differentiation is most clearly manifested by a continuous increase in the total adenine nucleotide (AMP + ADP + ATP) and ATP content. The total adenine nucleotide content started to increase (by 26%) at the 5th day of differentiation and was further increasing (by 40% at the 8th, by 100% at the 10th and by 117% at the 12th day as compared with control cells). There was no statistically significant difference in total adenine nucleotide content between days 10 and 12. The content of AMP and ADP was statistically significantly increasing from the 8th day and of ATP from the 5th day of differentiation (Fig. 6).

The ATP / ADP ratio is one of the important markers of ATP consumption intensity. Upon differentiation, the ATP / ADP ratio was significantly decreasing starting from the 8th day of differentiation by 13%, at day 10 by 19%, day 12 by 33%, as compared with control. The decrease of the ATP / ADP ratio indicates that from the 5th day of differentiation, along with an increase in the total content of adenine nucleotides, substantial changes of energy metabolism in the cell occurred. The ATP consumption increased, possibly because of an increased energy demand for the accelerated synthesis of proteins and structural elements peculiar to differentiated cells. Additional studies are needed to identify the processes most demanding as regards energy and ATP consumption.

Our study confirms and provides more detailed evidences for the suggestion of other authors that stemness may be estimated using certain functional mitochondrial characteristics [8], indicating that along with the numerous changes in cell infrastructure, including the redistribution of mitochondria, an increase in the unusually low mitochondrial NADH content may serve for the noninvasive fluorescence measurement based monitoring of the development of complex differentiation processes. Thus, we suggest that an increase in NAD(P)H fluorescence may be useful for a non-invasive detection of the onset of myoblast differentiation.

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MIOGENINIŲ KAMIENINIŲ LĄSTELIŲ POKYČIAI DIFERENCIACIJOS METU

Santrauka

Griaučių raumenų mioblastai yra taikomi ląstelių terapijai ir pažeistam miokardui regeneruoti. Norint parinkti audinių atkūrimo optimalias sąlygas, svarbu nustatyti tinkamus diferenciacijos žymenis. Mes įvertinome diferenciacijos sukeltus ląstelių dydžio, mitochondrijų pasiskirstymo ląstelėje, adenino bei piridino nukleotidų kiekio ir NAD(P)H fluorescencijos pokyčius triušio mioblastuose. Diferenciacijos metu didėjo didelių ląstelių frakcijos kiekis. Mitochondrijų išsidėstymas nuo perinuklearinio nediferencijuotose ląstelėse keitėsi į tolygų išsidėstymą diferencijuotų ląstelių citoplazmoje. Pastebėta išskirtinė nediferencijuotų mioblastų savybė – neįprastai maža NAD(P)H fluorescencija, kuri, sukėlus ląstelių diferenciaciją, didėjo dėl metabolinių ir fiziologinių pokyčių, tačiau nepriklausė nuo ląstelių didėjimo. Patikimas NAD(P)H kiekio padidėjimas chromatografiškai buvo nustatytas po 8 diferenciacijos dienų, nors NAD(P)H fluorescencija padidėjo 42 % jau po 3 dienų ir 2,7 karto – po 5 diferenciacijos dienų, lyginant su kontrole. Nustatėme, kad ankstyvus fluorescencijos pokyčius lemia mitochondrijų NADH kiekio didėjimas, nors ilgesnės diferenciacijos metu ląstelėse padidėja ir NADPH kiekis. Bendras adenino nukleotidų (ATP, ADP, AMP) kiekis ląstelėse diferenciacijos metu didėjo, bet ATP ir ADP santykis mažėjo. Tyrimų rezultatai leidžia teigti, kad NAD(P)H fluorescencijos didėjimas gali būti vertingas neinvazyvus metodas vertinant mioblastų diferenciacijos pradžią ir eigą.

Raktažodžiai: miogeninės kamieninės ląstelės, diferenciacija, NAD(P)H fluorescencija