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Nuclear localization of aldolase A correlates with cell proliferation

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ABSTRACT

Muscle fructose 1,6-bisphosphate aldolase (ALDA) is a glycolytic enzyme which may localize both in nuclei and cytoplasm of cells, however its role in the nuclei is unclear. Here, we demonstrate the links between subcellular localization of ALDA and the cell cycle progression as well as the availability of energetic substrates. Results of our studies indicate that nuclear localization of ALDA correlates with the proliferative activity of the cells and with the expression of Ki-67, a marker of proliferation, both in the KLN-205 (mouse lung cancer cells) and human squamous cell lung cancer cells (hSCC). Chemically-induced block of cell cycle entry in S phase and the inhibition of transcription stimulate removal of ALDA from cells nuclei suggesting that nuclear ALDA is involved in cells proliferation. On the other hand, subcellular distribution of the enzyme also depends on the stress and pro-survival signals mediated by the Akt and the p38 pathways and, in non-proliferating cells, on the availability of glucose and lactate. The results presented here point to ALDA as a factor involved in the regulation of cells proliferation.

1. Introduction

Fructose 1,6-bisphosphate aldolase (ALD; EC 4.1.2.13) catalyses the reversible cleavage of fructose 1,6-bisphosphate to dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate [1]. In mammalian tissues, three aldolase isozymes are expressed: ALDA (expressed primarily in muscles), aldolase B (mainly expressed in liver) and ALDC (expressed predominantly in neuronal tissues) [1,2]. However, in most of tissues the simultaneous expression at least of two isozymes is observed [1,3]. Cancer tissues may express all aldolase isozymes, but ALDA is the most commonly expressed [4,2,5].

Over the years evidence has accumulated that ALDA with other glycolytic enzymes may form metabolic complex [6,7] to ensure the effective flux of intermediates through the glycolysis. Nonetheless, the nuclear localization of aldolase was also observed in many cells [8–11,3] and it was shown that aldolase may associate with nucleic acids [8,12].

Previously, we demonstrated that ALDA localized in nuclei of retinal proliferating cells [13]. In the present study we demonstrated that nuclear localization of ALDA in mouse cultured lung cancer cells (the KLN-205 line) and human squamous cell lung cancer (hSCC) correlated with the rate of cells proliferation and the nuclear localization of Ki-67, the protein expressed solely during cell cycle [14–16]. We also show that down-regulation of ALDA expression with antisense oligonucleotide resulted in the reduction of proliferative activity of cancer cells and this observation is in line with the latest findings of Lew and Tolan [17] who have found that silencing of aldolase expression inhibited cell proliferation. In the current report we also demonstrated that blocking of cell cycle progression through S phase and the inhibition of transcription promoted the removal of ALDA from the nuclei. On the other hand, we found that in non-proliferating cancer cells, the subcellular distribution of the enzyme was regulated by energy metabolism substrates: glucose, lactate and glutamine.

The results presented in the manuscript suggest that nuclear ALDA may be involved in a regulation of transcription of genes engaged in the cell cycle progression.

2. Materials and methods

2.1. Chemicals

Polyester wax was from Science Services (Munchen, Germany). Anti-Ki-67 immunoglobulins (NCL-Ki-67p) and Nunc LockWell™ Maxisorp C8 StarWell plates were from Biokom (Janki, Poland). Rapamycin and Akt inhibitor IV were obtained from Santa Cruz Biotechnology Inc.(Santa Cruz, California, USA). Rabbit anti-Akt1/PKB (phospho S473) antibodies were obtained from Spring Bioscience (Pleasanton, California, USA). SuperSignal West Pico Chemiluminescent Substrate was from Thermo Scientific. All other reagents were

Abbreviations: hSCC, human squamous cell lung cancer; ALDA, muscle aldolase isozyme; ALDC, brain aldolase isozyme; APC, aphidicolin; ConA, concanavalin A; ACD, actinomycin D; LNA-oligo, locked nucleic acid antisense oligonucleotide

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from Sigma-Aldrich (Poznan, Poland) and were of the cell culture grade.

Histologically proven human squamous cell lung cancer (hSCC) tissue was obtained from Lower Silesian Pulmonary Center (Wroclaw, Lower Silesia, Poland) in agreement with the rules of the Scientific Research Ethical Committee. Rabbit muscle aldolase was purified according to Penhoet et al. [1]. Mouse polyclonal antiserum against muscle aldolase was produced as described previously [11]. The specificity of the immunoglobulins against aldolase was confirmed by immunoblotting [18] and preabsorption experiments [19] (Supplementary material; Figs. S1, S2).

2.2. Cell culture

All the cell lines were cultured at 37 °C in a humidified atmosphere with 5% CO_2 and maintained using standard tissue culture techniques. Mouse squamous cell carcinoma cell line (the KLN-205) was obtained from Sigma-Aldrich (Poznan, Poland). The primary culture of explant-derived human lung cells (the hSCC) was prepared according to Freeman and Hoffman [20]. Immediately after surgery the histologically proven Non Small Cell Lung Cancer tumor fragments were put into Hank's Balanced Salt solution, dissected with scissor into 1 mm^3 sections, and put into culture dishes coated with matrigel. The primary cultures of NSCLC cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with glucose (1 g/L) and sodium pyruvate (0.11 g/L). To avoid fibroblasts outgrowth of cancer primary cultures, L-valine was substituted to D-valine (0.094 g/L) [21]. Additionally, the medium was supplemented with 2 mM glutamine, 1% non-essential amino acids (NEAA), penicillin (100 U/mL), streptomycin (0.1 mg/mL) and 10% fetal bovine serum (FBS). For immunofluorescent localization of aldolases and Ki-67, explant-derived hSCC cells were subcultured, by trypsinization, into a new plates without matrigel, and cultured in DMEM with L-valine, supplemented with glutamine, NEAA, FBS and antibiotics (in concentrations described above). In order to verify the purity of explant-derived hSCC line culture, the cells were immunostained for cytokeratin-7, a marker of cancer cells [22] (Supplementary material; Fig. S3).

The KLN-205 cells were cultured in Eagle's Minimum Essential Medium (without sodium pyruvate) supplemented with 2 mM glutamine, 1% non-essential amino acids, penicillin (100 U/mL), streptomycin (0.1 mg/mL), glucose (1 g/L) and 10% fetal bovine serum (FBS).

The serum starvation experiment as well as aphidicolin (APC) and concanavalin A (ConA) treatments of the KLN-205 cultures were performed according to Mamczur et al. [19]. To block the transcriptional activity of cells, the KLN-205 cells were incubated for 20 h with 2.5 μg/mL or 10 μg/mL of actinomycin D as it was described by Bensaude [23].

To investigate the effect of energy substrates on ALDA subcellular distribution in non-proliferating cells, serum deprived the KLN-205 cells (G0 phase cells) were cultured for 20 h in DMEM with L-valine (0.094 g/L) and sodium pyruvate (0.11 g/L) supplemented with NEAA, FBS and antibiotics (in concentrations described above) as well as with glucose (5.5 mM) and/or glutamine (2 mM) and/or lactate (20 mM).

2.3. Activity measurement

Aldolase activity was assayed spectrophotometrically as described previously [24], with slight modifications. One milliliter of the aldolase assay mixture contained: 1 mM fructose 1,6-bisphosphate, 0.2 mM NADH, 5 U triose 3-phosphate isomerase, 5 U glycerol 3-phosphate dehydrogenase in the buffer (50 mM Tris, 0.1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4, 37 °C). One unit of enzyme activity is defined as the amount of the enzyme that catalyses the formation of 1 μmol of product per minute.

To determine aldolase activity in whole cells homogenates, the KLN-205 cells were trypsinized, washed with PBS (phosphate-buffered saline: 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4; pH 7.5, RT) and homogenized in a buffer (50 mM Tris, 250 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.014 mg/mL leupeptin; pH 7.4, 4 °C). Then, the homogenate was centrifuged (20 min, 20,000*g*, 4 °C) and the supernatant was assayed for the enzyme activity and protein concentration.

Protein concentrations were determined spectrophotometrically using Bradford Reagent (Sigma-Aldrich, Poznan, Poland), according to the manufacturer's requirements.

All spectrophotometric measurements were performed with an Agilent 8453 diode array spectrophotometer. The isolation of nuclei and cytosol from the KLN-205 cells was performed with CelLytic™ NuCLEAR™ Extraction Kit according to manufacturer's requirements (Sigma-Aldrich). The determination of glucose 6-phosphate dehydrogenase activity (the marker of the cytosolic fraction), and immunoblot for lamin A (nuclear fraction marker) was performed to confirm the purity of subcellular fractions (Supplementary material; Table S1, Fig. S4).

2.4. Immunofluorescence

hSCC sections and cultured cells were prepared for immunofluorescent studies as describe previously [19], with slight modifications. The cells and the tissue sections were incubated (overnight, at 4 °C) with mouse polyclonal anti-aldolase A (1:100) and either with rabbit polyclonal anti-Ki-67 (1:1000) or rabbit polyclonal anti-aldolase C (1:100) immunoglobulins, followed by the incubation (30 min. at RT) with fluorophore-labeled secondary antibodies: goat anti-rabbit-FITC (1:400–1:2,000) and goat anti-mouse-TRITC (1:500–1:2,000). For the simultaneous detection of ALDA and phospho-Akt (pAkt) in the KLN-205, the cells were incubated with polyclonal rabbit anti-Akt1/PKB (phospho S473) (1:100) and mouse polyclonal anti-aldolase A (1:100), followed by the incubation with the secondary antibodies (as above). The tissue sections and cultures were counterstained with DAPI (0.5 μg/mL, 5 min, RT) to visualize the nuclei. In negative controls, the primary antibodies were omitted (Supplementary material; Fig. S5). Additionally, in order to check whether the use of different secondary antibodies may affect aldolase A localization pattern, the cells were stained with mouse polyclonal anti-aldolase A antibodies (1:100) and either with goat anti-mouse-FITC (1:1000) or goat anti-mouse-TRITC (1:2000) secondary antibodies (Supplementary material; Fig. S6).

2.5. Down-regulation of ALDA gene expression

Down-regulation of ALDA expression was performed with the use of locked nucleic acid antisense oligonucleotide (LNA-oligo) in the absence of transfection reagent [25]. The LNA-oligo complementary to the sequence of mouse ALDA mRNA (GenBank: Y00516.1) was synthesized and purified by Metabion International AG (Martinsried, Germany). This oligonucleotide is the 14-nucleotide long gapmer of the following sequence: 5′-^{m}CGgtgagcgatgTC^{m}-3′ (capital letters — LNA, lowercase letters — DNA, ^{m} — methylcytosine) and phosphorothioate internucleoside linkages. To stimulate the decrease of ALDA expression in the KLN-205 cell culture, the cells were seeded at low density (1000 cells/cm^2) and cultured for 24 h (as described above). Then, the medium was replaced by DMEM, supplemented with 2 mM glutamine, penicillin (100 U/mL), streptomycin (0.1 mg/mL), 1% NEAA, 10% FBS and 5 μM LNA-oligo, and cultured for the next 6 days. The experiment was performed in the absence and in the presence of glucose (5.5 mM).

2.6. Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was performed according to Lukong et al. [26], with modifications. The protein extracts

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