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Sperm-specific glyceraldehyde-3-phosphate dehydrogenase is expressed in melanoma cells

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ABSTRACT

Sperm-specific glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDS) is normally expressed only in sperms, but not in somatic tissues. Analysis of the expression of GAPDS mRNA in different cancer cell lines shows that the content of GAPDS mRNA is enhanced in some lines of melanoma cells. The purpose of the study was to assay melanoma cells for the expression of protein GAPDS. Three different lines of melanoma cells were investigated. By data of Western blotting, all investigated cells contain a 37-kDa fragment of GAPDS polypeptide chain, which corresponds to the enzyme GAPDS lacking N-terminal amino acid sequence that attaches the enzyme to the cytoskeleton of the sperm flagellum. The results suggest that GAPDS is expressed in melanoma cells without N-terminal domain. The immunoprecipitation of proteins from melanoma cell extracts using rabbit polyclonal antibodies against native GAPDS allowed isolation of complexes containing 37-kDa subunit of GAPDS and full-length subunit of somatic glyceraldehyde-3-phosphate dehydrogenase (GAPD). The results indicate that melanoma cells express both isoenzymes, which results in the formation of heterotetrameric complexes. Immunocytochemical staining of melanoma cells revealed native GAPDS in the cytoplasm. It is assumed that the expression of GAPDS in melanoma cells may facilitate glycolysis and prevent the induction of apoptosis.

1. Introduction

Genes expressed both in normal testes and in malignant tumors (Cancer/testes associated (CTA) genes) form a gene group that is being intensively investigated since it is important for the understanding of the mechanisms of the tumor genesis and for the search of new methods of diagnostics and treatment of the cancer. Nearly 100 CTA genes have been already identified, and their number is constantly growing. Nevertheless, a question that is constantly raised is whether the expression of testes-specific genes in cancer tumors is a random process accounted for the instability of the genome in cancer cells, or it is a specific feature of the growing tumor that is necessary for its development.

One of the specific features of cancer cells is the change in their metabolism. In normal cells, the functioning of the mitochondrial system of oxidative phosphorylation in the presence of oxygen results in the inhibition of glycolysis (Pasteur effect), but this effect is absent in cancer cells. Moreover, cancer cells are characterized by not only high intensity of glycolysis (anaerobic pathway of metabolism), but also by inhibition of respiration (Crabtree effect [1]). There is no unambiguous interpretation of these effects and their mechanisms, but it is assumed that the compartmentalization of glycolytic enzymes and interactions between them, as well as specific features of catalytic and regulatory mechanisms must be of importance for the regulation of the balance between glycolysis and oxidative phosphorylation.

It may be assumed that the expression of sperm-specific glycolytic enzymes in cancer cells could change the regulation of glycolysis and the coupled metabolic pathways. The expression of one of glycolytic enzymes, sperm-specific lactate dehydrogenase C was revealed in different human cancer tumors, and this enzyme was suggested to be responsible for the constitutive activation of the anaerobic pathway in cancer cells [2].

It should be noted that sperm-specific isoforms of glycolytic enzymes significantly differ from corresponding somatic isoenzymes in a number of catalytic and regulatory parameters, including stability and compartmentalization. The main function of these

Abbreviations: GAPDS, sperm-specific isoform of glyceraldehyde-3-phosphate dehydrogenase; GAPD, somatic isoform of glyceraldehyde-3-phosphate dehydrogenase; dN-GAPD, recombinant human sperm-specific glyceraldehyde-3-phosphate dehydrogenase lacking 68 N-terminal amino acid residues.

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enzymes is to supply energy for the contractive elements of the principal part of the sperm flagellum providing progressive movement of the sperm (mitochondria were shown to provide only 20% of the energy [3]). Some glycolytic enzymes are firmly attached to the cytoskeleton of the flagellum (so called fibrous sheath [4]).

Sperm-specific glyceraldehyde-3-phosphate dehydrogenase (GAPDS) significantly differs from the somatic isoenzyme GAPD. GAPDS possesses an additional N-terminal sequence (72 amino acid residues in human) providing the attachment of the enzyme to the fibrous sheath of the sperm flagellum [5,6] and exhibits an enhanced stability [7] compared to the somatic enzyme GAPD. Besides, the amino acid sequence of GAPDS lacks the motifs that are responsible for the translocation of the somatic enzyme GAPD through the nuclear membrane [8]. Thus, GAPDS cannot be involved in the apoptosis, the process controlled by the somatic GAPD [9,10]. We assumed that GAPDS could be expressed in cancer cells, and the emergence of the protein with properties that differed from those of the somatic isoenzyme could significantly affect their metabolism and proliferation.

We analyzed information on the expression of GAPDS mRNA in 15322 samples that is available in the ArrayExpress Database (www.ebi.ac.uk/arrayexpress, accession numbers E-TABM-185, E-GEOD-2109, E-MTAB-37, E-MTAB-62, E-GEOD-7127, E-GEOD-10843 and E-GEOD-7307). By these data, the expression of GAPDS mRNA in most cancer cell lines, as well as in somatic tissues is virtually absent. At the same time, in some investigated melanoma cell lines, the content of GAPDS mRNA is close to its content in the testes. Consequently, the sperm-specific enzyme GAPDS could be found in melanoma cells.

In the present work, using different methods, we identified sperm-specific glyceraldehyde-3-phosphate dehydrogenase in several lines of human melanoma cells. Thus, subsequent investigation of this protein could be useful for the understanding of the role of GAPDS in the altered metabolism and proliferation of cancer cells.

2. Materials and methods

Melanoma cell lines MelIL, MelKor, and MelP were obtained previously during the collaborative work with Blokhin Cancer Research Center of Russian Academy of Medical Sciences and Petrov Research Institute of Oncology [11]. Primary cultures were isolated from metastatic tumors of patients suffering from melanoma. All cell lines were cultivated in a RPMI-1640 medium (HyClone, USA) with the addition of 10% fetal bovine serum (HyClone), 2 mM L-glutamine (HyClone), 100 U/ml penicillin (Sintez, Russia), and 100 μg/ml streptomycin (Biokhimik, Russia) under 7% CO_2, 95% humidity and 37 °C.

Recombinant human sperm-specific glyceraldehyde-3-phosphate dehydrogenase with the deletion of 68 N-terminal amino acids (dN-GAPDS) was obtained as previously described [7].

Glyceraldehyde-3-phosphate dehydrogenase from rabbit skeletal muscles was isolated by Scopes method [12] followed by gel filtration on a Sephadex G-100 column.

2.1. Preparation of cell extracts

Cells were harvested from the Petri dishes using 300 μl of buffer (20 mM Tris–HCl, 150 mM NaCl, 0.1% Triton X-100, 1 mM PMSF). The cell suspension was sonicated and centrifuged (14,000*g*, 30 min). The resulting supernatant was used for immunoprecipitation and Western blotting as described below.

2.2. Immunoprecipitation experiments

Rabbit polyclonal antibodies against native GAPDS obtained as described in [13] were immobilized on Protein G Sepharose™ 4 Fast Flow (GE Healthcare, Great Britain). The gel of Protein G Sepharose (50 μl) was washed 3 times in an Eppendorf tube with buffer A (20 mM Tris, 150 mM NaCl, pH 7.5). Then the antibodies (25 μl of 5 mg/ml solution in buffer A were added to the Sepharose gel, and the suspension was incubated for 30 min at 4 °C under gentle shaking. The Sepharose with bound antibodies was washed with buffer A until no protein was detected in the eluate. Extracts of melanoma cells were prepared as described above. The extract (~300 μl of a ~2 mg/ml solution) was diluted with 700 μl of buffer A, and 300 μl of the resulting solution was added to the Protein G Sepharose containing bound antibodies. The suspension was incubated for 45 min at 4 °C under gentle shaking, and then washed until no protein was detected in the eluate. The washed Sepharose gel containing bound proteins was assayed by SDS PAGE or by Western blotting as described below.

2.3. Western blotting

SDS PAGE in 10% polyacrylamide gel was performed according to Laemmli [14]. Extracts were diluted twice with Laemmli 2× loading buffer containing 10% β-mercaptoethanol, and the samples were heated in boiling water for 15 min. Each sample loaded into a well contained 10 μg of total protein in the case of cell extracts or 1–2 μg of purified proteins used as the markers. After electrophoresis, the proteins were transferred to a HYBOND-P PVDF membrane (GE Healthcare) (90 mA, 1 h). The membrane was blocked with PBST containing 5% dry skim milk for 1 h at room temperature. GAPDS was detected using rabbit polyclonal antibodies against denatured GAPDS (2 μg/ml in PBST containing 1% dry skim milk) obtained as described in [13]. Antibodies against rabbit IgG conjugated with horseradish peroxidase were used as the secondary antibodies (GE Healthcare). The proteins were visualized with an Immobilon Western kit (Millipore, USA) using a BioRad Universal Hood II device.

2.4. Immunocytochemical staining of melanoma cells

Human melanoma cells were grown on coverslips for 20 h and fixed with cold methanol for 10 min. The coverslips were washed twice with PBS and then incubated in PBST containing 5% skim dry milk (1 h, 20 °C). The samples were incubated with a solution (50 μg/ml in PBS containing 1% BSA) of rabbit polyclonal antibodies to native GAPDS [13]. Antibodies against rabbit IgG conjugated with Alexa Fluor® 488 Dye (Invitrogen Molecular Probes™, USA) (10 μg in PBST containing 1% skim dry milk) were used as the secondary antibodies. Nuclei were stained with DAPI (Sigma–Aldrich, USA). The cells were examined using a Leica DMR fluorescent microscope equipped with a Leica DC Camera (Leica Microsystems Wetzlar GmbH).

2.5. MALDI mass-spectrometry analysis

Mass-spectra were recorded using an UltrafleXtreme MALDI TOF mass spectrometer (BrukerDaltonics, Germany) equipped with a UV Nd laser in the positive ion reflectron mode. The accuracy of the monoisotopic mass measurements constituted 0.005% (50 ppm). Spectra were acquired over the *m/z* range of 700–4500. Proteins were searched in the NCBI protein database using a Mascot: peptide mass fingerprint program (www.matrixscience.com) allowing for a possible oxidation of methionine residues by oxygen and modification of cysteines by acrylamide. Protein identification was considered to be reliable ($p < 0.05$) if a score exceeds 87.

3. Results and discussion

Analysis of the expression of GAPDS mRNA in different cancer tissues showed an increased level of GAPDS mRNA in some

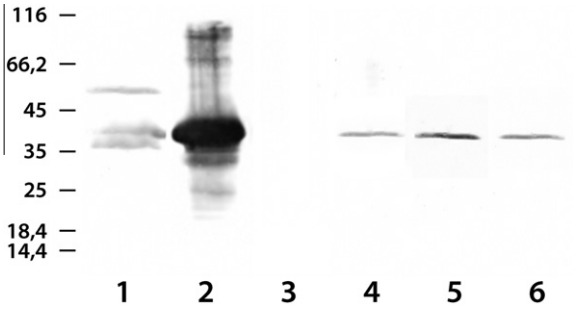

Fig. 1. Western blotting of melanoma cell extracts. The membrane was incubated in a solution of rabbit polyclonal antibodies against denatured dN-GAPDS; antibodies against rabbit IgG conjugated with peroxidase were used as the secondary antibodies: (1) sperm cell lysate; (2) purified dN-GAPDS; (3) purified rabbit muscle GAPD; (4–6) extracts of melanoma cells (lines Melll, MelP, and MelKor, respectively).

melanoma cell lines (ArrayExpress database, www.ebi.ac.uk/arrayexpress, accession numbers E-MTAB-37, E-MTAB-62, E-GEOD-10843 and E-GEOD-7127). This suggested that the sperm-specific enzyme GAPDS could be revealed in melanoma cells.

On the first stage of our investigations, we assayed three different lines of melanoma cells for expression of GAPDS by Western blotting of the corresponding cell extracts. To detect GAPDS, we used rabbit polyclonal antibodies interacting with denatured species of GAPDS obtained as described in [13]. As positive controls, we used sperm cells (Fig. 1, lane 1) and a preparation of recombinant sperm-specific glyceraldehyde-3-phosphate dehydrogenase without N-terminal domain (dN-GAPDS, Fig. 1, lane 2).

As seen in Fig. 1, lane 1, sperm cells yield a set of protein bands: a band of approximately 56 kDa corresponding to the full-length GAPDS [5] and shorter bands corresponding to the products of its proteolysis (37 and 36 kDa). Fig. 1, lane 2 shows a preparation of recombinant enzyme dN-GAPDS (~37 kDa). It is of importance that under the conditions employed, the used antibodies against GAPDS do not interact with the somatic enzyme GAPD (Fig. 1, lane 3), that is present in the analyzed extracts in significant amounts. In the tested lines of melanoma cells, we revealed a protein that interacted with the antibodies against human GAPDS (Fig. 1, lanes 4–6) with the molecular mass of approximately 37 kDa, which corresponded to the molecular mass of dN-GAPDS, but differed from that of the somatic enzyme GAPD (36 kDa).

This protein was detected neither in other somatic cells, nor in a number of cancer cells, this correlating with low expression of GAPDS mRNA in these tissues (Table 1). At the same time, in some melanoma cell lines the content of GAPDS mRNA exceeds its content in testes up to 4 times (Table 1), indicating the enhanced expression of GAPDS.

Thus, the analysis of GAPDS mRNA content in different cells points to a possibility of the expression of GAPDS in melanoma cells. Analysis of 3 different lines of melanoma cells revealed a protein with the subunit of ~37 kDa interacting with the antibodies against GAPDS. The revealed protein could be a fragment of the full-length protein GAPDS (56 kDa) that is characteristic for sperms. The molecular mass of the fragment is close to that of the recombinant GAPDS with the deleted N-terminal domain (see lanes 2 and 4–6 in Fig. 1). Such a fragment could be a result of the proteolysis of full-length GAPDS while preparing samples, as well as due to the proteolysis of the protein in the melanoma cells. It is also possible that GAPDS is expressed in melanoma cells without N-terminal domain: it is necessary for the attachment of the protein to the cytoskeleton of the sperm flagellum, but, while being expressed in *Escherichia coli* cells, it prevents normal protein folding. Deletion of the N-terminal domain yields soluble and catalytically active enzyme [7]. Analysis of mRNAs using the GenBank database showed that GAPDS without N-terminal domain is present in actively reproducing cells (embryonic and regenerating tissue cells) in some vertebrates (*Anolis carolinensis*) [15].

The presented results suggest that the revealed fragment of GAPDS could be characteristic for melanoma cells. However, these data give no information concerning the production of the native form of the enzyme, i.e. correctly folded and catalytically active protein. To reveal native GAPDS species in melanoma cells by immunoprecipitation, we used affinity-purified polyclonal antibodies interacting selectively with native form of GAPDS [13], which excluded the interactions with denatured GAPDS species. The experiments were performed with two of three mentioned melanoma lines, MelP and MelKor (Fig. 2). After the incubation of the protein G Sepharose-bound antibodies with the melanoma

Table 1
Analysis of different cell lines for expression of GAPDS by Western blotting. Data on the mRNA content are taken from ArrayExpress (www.ebi.ac.uk/arrayexpress, accession numbers E-MTAB-37, E-MTAB-62 and E-GEOD-7127). The expression level of GAPDS mRNA in testes is taken as one unit.

Cell line	Tissue or type of cells	Protein GAPDS (Western blotting)	GAPDS mRNA	GAPD mRNA
	Testes	+	1.0	7.21
	Fibroblasts	–	0.04	14.87
HEK293T	Embryonic kidney	–	0.04	8.16
SKOV-3	Ovarian cancer cells	–	0.02	6.39
HL60	Promyelocytic leukemia	–	0.04	9.72
LnCaP	Prostate adenocarcinoma	–	0.03	9.21
Raji	Burkitt lymphoma	–	0.05	15.08
K562	Erythromyeloblastoid leukemia	–	0.03	11.29
PC3	Prostate adenocarcinoma	–	0.05	12.11
Jurkat	T-cell leukemia	–	0.04	14.04
HT-1080	Fibrosarcoma	–	0.04	8.48
A549	Lung adenocarcinoma	–	0.04	13.41
MCF7	Breast adenocarcinoma	–	0.03	14.96
RKO	Colonic carcinoma	–	0.04	11.30
MelIL	Melanoma	+		
MelP	Melanoma	+	0.01–4*	7–14*
MelKor	Melanoma	+		

* Data spread are given for 63 melanoma cell lines (accession number GEOD-7127 in the ArrayExpress database).

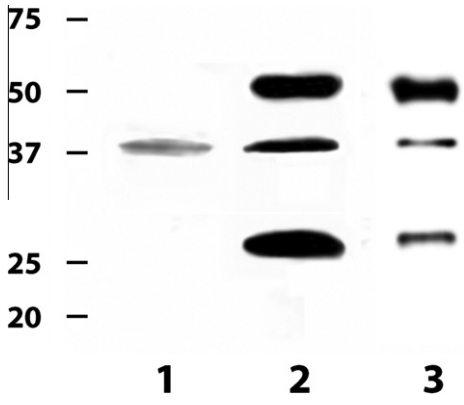

Fig. 2. Western blotting of the complexes obtained after the immunoprecipitation of proteins from the extract of melanoma cell MelP and MelKor: (1) purified dN-GAPDS; (2, 3) the complexes obtained after the incubation of Protein G Sepharose-bound antibodies against native GAPDS with extracts of melanoma cells MelP and MelKor, respectively. The membrane was stained with the polyclonal rabbit antibodies to denatured GAPDS (see Section 2 for details).

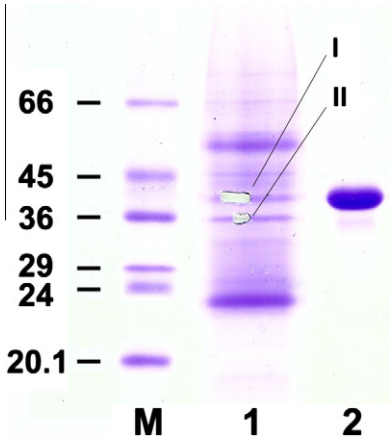

Fig. 3. SDS PAGE of the complex obtained by immunoprecipitation of proteins from MelP melanoma cell extract using polyclonal antibodies against GAPDS. (1) Protein G Sepharose-bound proteins; (2) purified dN-GAPDS.

cell extract, the formed complexes were analyzed by SDS PAGE with subsequent immunoblotting. To stain the membrane, antibodies against denatured GAPDS were used. The results are present in Fig. 2.

The presented data show that the complexes obtained after the incubation of the melanoma cell extracts with the Protein G Sepharose-bound antibodies against native GAPDS contain protein bands of ~37 kDa (Fig. 2, lanes 2 and 3). Besides, the complexes contain light and heavy chains of rabbit antibodies used in the immunoprecipitation (Fig. 2, lanes 2 and 3) that are also developed during the staining procedure since they interact with secondary antibodies. The molecular mass of the protein isolated from melanoma cell extracts interacting with anti-GAPDS antibodies is identical to the molecular mass of the proteins revealed in the original cell lysates (Fig. 1, lanes 4–6) and close to that of dN-GAPDS(Fig. 2, lane 1). These results support the idea concerning the expression in melanoma cells the fragment of GAPDS lacking its N-terminal domain, but maintaining its native structure.

Finally, the 37-kDa protein fragment interacting with anti-GAPDS antibodies isolated from melanoma cell extracts was identified using MALDI mass-spectrometry. The complex obtained after the incubation of Protein G Sepharose-bound antibodies against native GAPDS with MelP cell extract was analyzed by SDS PAGE.

As seen from Fig. 3, the isolated complex contains protein bands of ~50 and 25 kDa, which corresponds to the heavy and light chains of antibodies, and also protein bands of 36 and 37 kDa (lane 1). A sample of purified dN-GAPDS (37 kDa) is shown in Fig. 3, lane 2. The fragments of 37- and 36-kDa protein bands (I and II, respectively) were analyzed by MALDI mass-spectrometry analysis. The results are presented in Table 2.

Thus, it was supported that the 37-kDa protein revealed in the extracts of melanoma cells interacting with anti-GAPDS antibodies is a fragment of sperm-specific enzyme GAPDS. The sample II with the molecular mass of 36 kDa was identified as somatic GAPD. As seen from Fig. 3, the content of GAPD is close to that of GAPDS. Since the antibodies used for the immunoprecipitation are specific to GAPDS and do not interact with GAPD, the results suggest that the complex isolated from the MelP cell extract contains heterooligomeric forms of the enzyme composed of both GAPD and GAPDS subunits. Since usually GAPD exists as the tetramer where the interactions between the dimers are much weaker than between

Table 2
Mass-spectrometric protein identification results.

Protein index	Theoretical Mr/pI	MS/MS score	Protein coverage (%)	Accession	Protein description
1	37777/7.08	116	42	gi|238537990	Human sperm-specific glyceraldehyde-3-phosphate dehydrogenase
2	36030/8.57	186	60	gi|7669492	Human glyceraldehyde-3-phosphate dehydrogenase isoform 1

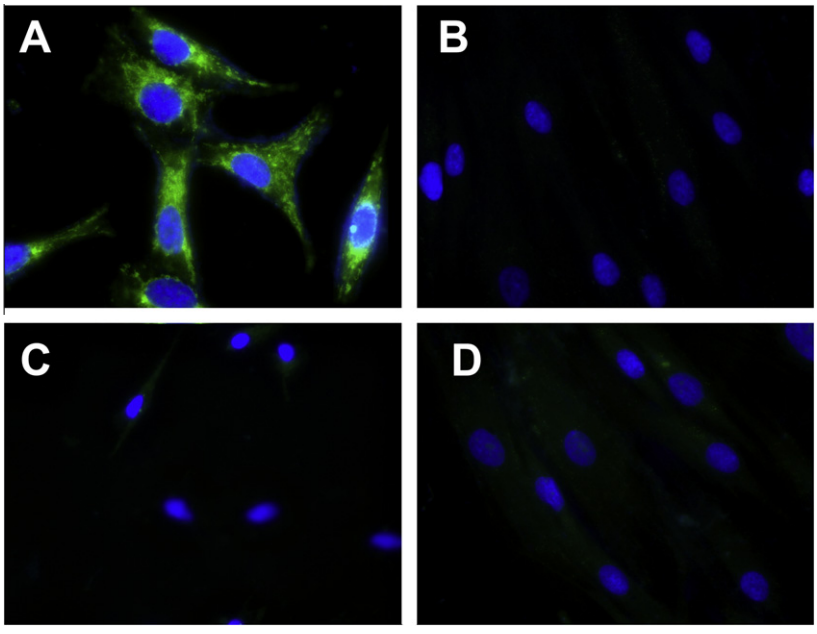

Fig. 4. Immunocytochemical staining of MelP melanoma cells (A, C). Fibroblasts were used as the negative control (B, D). The samples were treated with the rabbit antibodies against native GAPDS and then with secondary antibodies against rabbit IgG conjugated with Alexa Fluor® 488 Dye (A, B), or only with the secondary antibodies (C, D). Nuclei are stained with DAPI (A–D).

the monomers, and the ratio of two isoenzymes in the isolated complex is close to 1:1, the heterotetramers must contain one dimer of the somatic enzyme GAPD and one dimer of sperm-specific GAPDS.

Such hybrid forms of GAPDS were described previously: expression of rat GAPDS in *E. coli* cells resulted in the formation of tetramers containing of two types subunits: recombinant rat GAPDS and host GAPD [16]. Presumably, similar situation is observed in melanoma cells. It should be noted that formation of such hybrid forms may lead to significant consequences. It has been known that somatic enzyme GAPD is involved in cell apoptosis: dissociation of the protein into subunits results in the translocation of the protein into the nucleus [9]. GAPDS exhibits enhanced stability compared to GAPD [7], and it is possible that the formation of more stable hybrid form of the enzyme hamper the translocation of GAPD into the nucleus, which may play some role in the malignant transformation of melanoma cells.

On the final step of the study, we investigated localization of GAPDS in the melanoma cells. Fig. 4 demonstrates immunochemical staining of MelP melanoma cells with the use of rabbit polyclonal antibodies against native GAPDS. It is seen that GAPDS localized in the cytoplasm of the cells (Fig. 4A). Similar results were obtained with MelII and MelKor cell lines (data not shown). Fibroblasts that were used as the negative control showed no interaction with antibodies against GAPDS (Fig. 4B). For both type of cells, no staining was observed after the treatment with only secondary antibodies against rabbit IgG conjugated with the fluorescent dye (Fig. 4C and D).

Thus, we demonstrated that some lines of melanoma cells express sperm-specific enzyme GAPDS. At the same time, this enzyme was detected neither in somatic cells, nor in other investigated cancer cells (Table 1). It can be assumed that some properties of melanoma, in particular their high malignancy, may be due to the presence of GAPDS that exhibits a number of unusual properties compared to somatic isoenzyme GAPD. For example, GAPDS exhibits an enhanced stability [7], which allows the enzyme provide sperms with energy for progressive movement for several days without synthesis of novel portions of the protein, while the turnover of GAPD in somatic cells is 1–1.2 days [17]. Consequently, the expression of the additional stable isoenzyme GAPDS gives the melanoma cells an advantage in producing energy during glycolysis, which is the main source of energy in malignant cells.

It is also possible that more important function of GAPDS in melanoma cells is a disturbance of the induction of apoptosis. It is well known that somatic GAPD is involved in apoptosis. During this process, monomeric and denatured species of this enzyme are passively transported from the cytoplasm to the nucleus, and after additional unfolding and exposition of the nuclear export signal (NES), denatured forms are translocated back into the cytoplasm [18]. The passive transport of the more stable and, consequently, less prone to dissociation isoenzyme GAPDS and hybrid GAPD-GAPDS complexes to the nucleus must be hampered. Besides, GAPDS does not possess signal sequences for nuclear export [8], and consequently, cannot be involved in the induction of apoptosis.

We suppose that the expression of GAPDS in melanoma cells may alter their metabolism (first of all, efficiency of glycolysis) and prevent the induction of apoptosis. Besides, GAPDS may serve as a new marker for melanoma cells. This may be of special importance if the expression of GAPDS correlates with the extent of the malignancy of the tumor, but this matter needs special investigation.

Acknowledgments

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