FEBS Letters 582 (2008) 3525–3530

Enhanced glycolysis induced by mtDNA mutations does not regulate metastasis

Kaori Ishikawa[a,b,1], Osamu Hashizume[a,1], Nobuko Koshikawa[c], Sayaka Fukuda[a], Kazuto Nakada[a], Keizo Takenaga[d], Jun-Ichi Hayashi[a,*]

[a] *Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan*
[b] *Japan Society for the Promotion of Science (JSPS), 8 Ichiban-cho, Chiyoda-ku, Tokyo 102-8472, Japan*
[c] *Division of Chemotherapy, Chiba Cancer Center Research Institute, 666-2 Nitona, Chuo-ku, Chiba 260-8717, Japan*
[d] *Shimane University Faculty of Medicine, 89-1 Enya-cho, Izumo, Shimane 693-8501, Japan*

Received 4 August 2008; accepted 10 September 2008

Available online 19 September 2008

Edited by Veli-Pekka Lehto

Abstract **We addressed the issue of whether enhanced glycolysis caused by mtDNA mutations independently induces metastasis in tumor cells using mtDNA transfer technology. The resultant trans-mitochondrial cybrids sharing the same nuclear background of poorly metastatic carcinoma P29 cells, P29mtA11 and P29mtΔ cybrids, possessed mtDNA with a G13997A mutation from highly metastatic carcinoma A11 cells and mtDNA with a 4696 bp deletion mutation, respectively. The P29mtΔ cybrids expressed enhanced glycolysis, but did not express ROS overproduction and high metastatic potential, whereas P29mtA11 cybrids showed enhanced glycolysis, ROS overproduction, and high metastatic potential. Thus, enhanced glycolysis alone does not induce metastasis in the cybrids.**

Keywords: Pathogenic mtDNA mutation; mtDNA transfer technology; Trans-mitochondrial cybrid; Enhanced glycolysis; The Warburg effect; ROS overproduction; Metastasis

1. Introduction

Involvement of mtDNA mutations and the resultant mitochondrial respiration defects in tumor development have been suggested based on the evidence that most chemical carcinogens bind preferentially to mtDNA rather than to nuclear DNA [1–3]. Moreover, somatic mutations in mtDNA are accumulated preferentially in tumor cells rather than in normal cells of the same subjects [4,5], and many subsequent studies supported high frequencies of homoplasmic mutations in mtDNA of tumors [6–9].

On the contrary, our previous studies showed that mtDNA mutations were not involved in tumor development of cultured mouse [10,11] and human cells [12,13] using trans-mitochondrial cybrids obtained by mtDNA transfer between normal and tumor cells. The possibility that these observations represent some specific tumor cases can be excluded, since there has been no statistical evidence for the association of pathogenic mtDNA mutations and tumor development in the patients with mitochondrial diseases expressing respiration defects due to the pathogenic mtDNA mutations. The possibility of the involvement of polymorphic mtDNA mutations in tumor development also can be excluded, since there has been no statistical evidence for the presence of maternal inheritance of tumor development in spite of the strictly maternal inheritance of mammalian mtDNA [14,15].

However, it is still possible that mtDNA mutations are involved in other processes besides the oncogenic transformation of normal cells to develop tumors, such as the malignant progression of tumor cells to develop metastatic potential. Our recent study addressed this issue by means of mtDNA exchange technology, and demonstrated that mtDNA mutations inducing complex I defects and resultant overproduction of reactive oxygen species (ROS) reversibly controlled malignant progression of tumor cells to develop metastatic potential [16]. However, considering that complex I defects simultaneously induce enhanced glycolysis under normoxia (the Warburg effect) and ROS overproduction, it is necessary to determine whether the Warburg effect also controls metastasis independently. In fact, recent reports demonstrated that up-regulation of glycolysis caused by mutations or epigenetic controls of nuclear-coded genes regulate tumor phenotypes by the induction of a pseudo-hypoxic pathway under normoxia [17–20].

To address this issue, we generated trans-mitochondrial P29mtΔ cybrids by introduction of ΔmtDNA4696 with a 4696 bp deletion mutation into low metastatic Lewis lung carcinoma P29 cells. The P29mtΔ cybrids expressed mitochondrial respiration defects and enhanced glycolysis under normoxia, but did not express ROS overproduction, providing proper cellular system to examine whether the Warburg effect alone can control metastasis.

2. Materials and Methods

2.1. Cell lines and cell culture

The mouse cell lines and their characteristics are listed in Table 1. The P29 cells originated from Lewis lung carcinoma (C57BL/6 mouse strain), and B82 cells are fibrosarcoma cells derived from the L929 fibroblast cell line (C3H/An mouse strain). Parental cells, ρ^0 cells, and the trans-mitochondrial cybrids were grown in normal medium [DMEM + pyruvate (0.1 mg/ml) + uridine (50 mg/ml) + 10% fetal bovine serum].

*Corresponding author. Fax: +81 298 53 6614.
E-mail address: jih45@sakura.cc.tsukuba.ac.jp (J.-I. Hayashi).

[1]These authors contributed equally to this work.

0014-5793/$34.00
doi:10.1016/j.febslet.2008.09.024

Table 1
Genetic characteristics of parent cells and their trans-mitochondrial cybrids

Cell lines[a]	Nuclear genotypes (genetic marker)[b]	mtDNAgenotypes	Fusion combination			Selection
			Nuclear donors	×	mtDNA donors	
Nuclear donors						
ρ^0P29	P29 (HAT^r, $BrdU^s$)	mtDNA-less				
mtDNA donors						
B82mtP29	B82 (HAT^s, $BrdU^r$)	Wild type	ρ^0B82	×	en[c]P29	BrdU + UP^-
B82mtA11	B82 (HAT^s, $BrdU^r$)	G13997A	ρ^0B82	×	enA11	BrdU + UP^-
B82mtΔ	B82 (HAT^s, $BrdU^r$)	ΔmtDNA4696	ρ^0B82	×	platelets	UP^-
Trans-mitochondrial cybrids						
P29mtP29	P29 (HAT^r, $BrdU^s$)	Wild type	ρ^0P29	×	enB82mtP29	HAT + UP^-
P29mtA11	P29 (HAT^r, $BrdU^s$)	G13997A	ρ^0P29	×	enB82mtA11	HAT + UP^-
P29mtΔ	P29 (HAT^r, $BrdU^s$)	ΔmtDNA4696	ρ^0P29	×	enB82mtΔ	HAT + UP^-

[a]As mtDNA donors, we used B82mtP29, B82mtA11, and B82mtΔ cybrids shearing the same nuclear background of B82 cells for excluding variations of nuclear-coded cytoplasmic factors in mtDNA donors. B82mtP29 cybrids carrying nuclear DNA from B82 cells and mtDNA from P29 cells were obtained by fusion of ρ^0B82 cells with enucleated P29 cells and subsequent cultivation in the selection medium with BrdU and UP^-. ρ^0B82 cells can survive in the selection medium with BrdU due to their lacking thimidine kinase activity, and cannot survive in the selection medium without uridine and pyruvate (UP^- medium) due to their lacking mtDNA. Thus, BrdU and UP^- eliminate unenucleated P29 cells and unfused ρ^0B82 cells, respectively, and allow exclusive growth of the B82mtP29 cybrids. B82mtΔ cybrids carrying nuclear DNA from B82 cells and ΔmtDNA4696 were obtained by fusion of ρ^0B82 cells with platelets from mito-mice carrying ΔmtDNA4696 [23] in the UP^- selection medium. As G13997A mtDNA donors, we used B82mtA11 cybrids obtained in our previous work [16].
[b]All the mtDNA donors sharing the B82 nuclear background lacking thymidine kinase activity cannot survive in the presence of a hypoxanthine/aminopterin/thymidine (HAT). On the contrary, nuclear donors ρ^0P29 cells can grow in the HAT selection medium due to their processing thimidine kinase activity, but not in UP^- selection medium due to their complete respiration defects by mtDNA depletion. Thus, HAT and UP^- allow exclusive growth of the P29mtP29, P29mtA11, and P29mtΔ cybrids.
[c]en Represents enucleated.

2.2. Isolation of trans-mitochondrial cybrids

We isolated ρ^0 cells by treating parental cells with 1.5 mg/ml ditercalinium (DC), an antitumor bis-intercalating agent [21]. Complete depletion of mtDNA was confirmed by PCR analysis. Enucleated cells of the mtDNA donor were prepared by their pretreatment with cytochalasin B (10 μg/ml) for 10 min and centrifugation at $12000 \times g$ for 30 min. Resultant cytoplasts were fused with ρ^0 cells by polyethylene glycol. Trans-mitochondrial cybrids were isolated in the selection medium that allows exclusive growth of the cybrids (see Table 1).

2.3. Genotyping of mtDNAs

Transfer of mtDNAs in the cybrids was confirmed by RFLP analysis of the PCR products and Southern blot analysis. For recognition of the G13997A mutation, a 147-bp fragment containing the 13997 site was amplified by PCR. The nucleotide sequences from n.p. 13963 to 13996 (CCCACTAACAATTAAACCTAAACCTCCATActTA, small letters indicate the mismatch site) and n.p. 14109 to 14076 (TTCATGTCATTGGTCGCAGTTGAATGCTGTGTAG) were used as oligonucleotide primers. Combination of the PCR-generated mutation with the G13997A mutation creates a restriction site for *Afl* II, and generates 114-bp and 33-bp fragments on *Afl* II digestion. The restriction fragments were separated in 3% agarose gel. To estimate the proportion of ΔmtDNA, we carried out Southern blot analysis. Total DNAs (3 μg) extracted from cybrids were digested with the restriction enzyme *Xho* I. Restriction fragments were separated in 0.8% agarose gel, transferred to a nylon membrane, and hybridized with alkaline phosphatase-labeled mouse mtDNA probes (n.p. 1751–3803). Probe labeling and signal detection were carried out as described in the protocols of the AlkPhos Direct (GE Healthcare, Buckinghamshire, UK). For quantification of ΔmtDNA, we use the NIH IMAGE program.

2.4. Biochemical measurement of respiratory enzyme activities

Cells in log-phase growth were harvested, and the respiratory complexes were assayed as described before [22]. Briefly, NADH and cytochrome *c* (oxidized form) were used as substrates for estimation of complexes I + III activity, and the reduction of cytochrome *c* was monitored at 550 nm. For estimation of complexes II + III activity, sodium succinate and cytochrome *c* (oxidized form) were used as substrates, and reduction of cytochrome *c* was monitored at 550 nm. For estimation of complex IV activity, cytochrome *c* (reduced form) was used as substrates, and the enzyme activity was determined by monitoring the oxidation of cytochrome *c* at 550 nm.

2.5. Measurement of ROS production

ROS generation was detected with mitochondrial superoxide indicator MitoSOX-RED (Invitrogen, Carlsbad, CA, USA). Cells were incubated with 5 μM MitoSOX-RED for 10 min at 37 °C in serum-free DMEM, washed twice with Dulbecco's phosphate-buffered saline (DPBS), and then immediately analyzed with a FACS can flow cytometer (Becton Dickinson, Mountain View, CA, USA).

2.6. SDS–PAGE and Western blotting

To detect MCL-1, cells were lysed on ice for 10 min in 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM PMSF, and protease inhibitor cocktail (Sigma–Aldrich, St. Louis, MO, USA). After centrifugation at $10000 \times g$ for 10 min at 4 °C, the supernatant was used as a sample. Proteins were resolved by SDS–PAGE under reducing conditions. The resolved proteins were transferred electrophoretically to a nitrocellulose membrane. After incubation with 5% dry milk in TBS-T [150 mM NaCl, 50 mM Tris–HCl (pH 7.4), 0.05% Tween 20] for at least 1 h at room temperature, the membrane was incubated with polyclonal anti-MCL-1 antibody (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA, USA) for 1 h at room temperature, washed extensively with TBS-T, and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG. Proteins were detected using ECL Western blotting detection reagents (GE Healthcare). For loading controls, the membrane was stripped, and subsequently incubated with monoclonal anti-β-actin antibody (Sigma–Aldrich) followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG.

2.7. Measurement of the concentration of lactate in the cell medium

Cells were seeded at 5×10^4 cells/well of a 6-well plate and cultured for 24 h. The amounts of lactate in the cell medium were estimated using an F-kit L-Lactic acid (Roche, Basel, Switzerland).

2.8. Assays of metastatic potential

To test experimental metastatic potential, 5×10^5 cells/100 μl PBS were injected into the tail vein of 6-week-old male C57BL/6 mice (CLEA Japan, Tokyo, Japan). The mice were sacrificed 18 days later, and their lungs were removed. The lungs were fixed in the Bouin's solution, and parietal nodules were counted.