Effects of enhanced glycolysis by a mitochondrial DNA deletion mutation on expression of metastasis.

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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 4,696bp 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.

Key words: pathogenic mtDNA mutations/ mtDNA transfer technology/ trans-mitochondrial cybrids/ 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 4,696bp 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].

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 12,000 × *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 13,997 site was amplified by PCR. The nucleotide sequences from n.p. 13,963 to 13,996 (CCCACTAACAATTAAACCTAAACCTCCATActTA, small letters indicate the mismatch site) and n.p. 14,109 to 14,076 (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. 1,751-3,803). 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 ºC in serum-free DMEM, washed twice with Dulbecco's phosphate-buffered saline (DPBS), and then immediately analyzed with a FACScan 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 10,000 × *g* for 10 min at 4 ºC, 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.

3. Results

3-1 Isolation of the mtDNA donors sharing the same nuclear background of B82 cells

This study examined whether the Warburg effect induced by mitochondrial respiration defects alone controls metastasis in mouse Lewis lung carcinoma cells. For this, we used mtDNA-less (ρ^0) P29 cells derived from poorly metastatic Lewis lung carcinoma P29 cells as nuclear DNA donors and mtDNA recipients (Table 1).

As mtDNA donors, we isolated trans-mitochondrial B82mtP29, B82mtA11, and B82mtΔ cybrids shearing the same nuclear background of B82 cells (Table 1). The 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 (Table 1). The B82mtΔ cybrids carrying nuclear DNA of B82 cells and ΔmtDNA4696 were obtained by fusion of ρ^0B82 cells with platelets from mito-mice carrying ΔmtDNA4696 [23]. The B82mtA11 cybrids isolated in our previous work [16] were obtained by fusion of ρ^0B82 cells with enucleated A11 cells.

These cybrids are appropriate to be used as mtDNA donors to ρ^0P29 cells by the following two reasons. One is that the cybrids sharing the B82 nuclear background can exclude the influence of variations of the nuclear-coded cytoplasmic factors on metastasis of the mtDNA recipient ρ^0P29 cells. Another is that the cybrids sharing B82

nuclear background lack thymidine kinase activity, and cannot survive in the presence of a hypoxanthine/ aminopterin/ thymidine (HAT). Thus, unenucleated mtDNA donor cybrids can be excluded in the selective medium with HAT (Table 1).

3-2 Isolation of trans-mitochondrial cybrids carrying mtDNA with pathogenic mutation

These mtDNA donor cybrids were used for obtaining the trans-mitochondrial cybrids, P29mtP29, P29mtA11, and P29mtΔ (Table 1). First, we isolated trans-mitochondrial P29mtΔ cybrids, possessing the nuclear genome of the P29 cells and mitochondrial genome of ΔmtDNA4696 with a 4,696bp deletion mutation, by the fusion of the ρ^0 P29 cells with enucleated B82mtΔ cybrids (Table 1). Our previous reports showed that accumulation of the ΔmtDNA4696 induced suppression of overall mitochondrial translation due to the large-scale deletion mutation including 5 mitochondrial tRNA genes from ΔmtDNA4696 [23, 24]. Therefore, we expected that the P29mtΔ cybrids with ΔmtDNA4696 would express enhanced glycolysis under normoxia, but not express ROS overproduction, probably due to overall mitochondrial respiration defects induced by ΔmtDNA4696. Thus, the P29mtΔ cybrids would provide an ideal system to examine whether enhanced glycolysis alone could induce metastasis in poorly metastatic P29 cells.

We also obtained trans-mitochondrial P29mtA11 and P29mt29 cybrids by the fusion of the ρ^0 P29 cells with enucleated B82mtA11 and B82mtP29 cybrids for using as positive and negative controls of metastasis, respectively (Table 1). Our recent report [16] isolated P29mtA11 and P29mtP29 cybrids by the fusion of ρ^0P29 with enucleated A11 cells and P29 cells, respectively, and showed that the P29mtA11 cybrids were highly metastatic and P29mt29 cybrids were poorly metastatic. However, it was possible that some cytoplasmic factors encoded by nuclear DNA may affect metastatic

potentials, since nuclear background of mtDNA donor A11 and P29 cells are different from each other. This study can exclude this possibility by the use of the B82mtA11 and B82mtP29 cybrids sharing the same nuclear background of B82 cells as mtDNA donors.

3-3 mtDNA genotypes and resultant phenotypes of respiratory function in the cybrids

Using PCR and Southern blot analyses, we carried out the mtDNA genotyping of the P29mtP29, P29mtA11, and P29mtΔ cybrids (Fig. 1A). The G13997A mutation in mtDNA from A11 cells creates an *Afl* II site in PCR products amplified using mismatch primers [16]. Therefore, we carried out *Afl* II digestion of their PCR products, and showed that mtDNA in P29mtA11 cybrids possessed a homoplasmic G13997A mutation, while mtDNA in P29mtP29 and P29mtΔ cybrids did not (Fig. 1A, upper panel). The ΔmtDNA4696 was identified by Southern blot analysis. The results showed that P29mtΔ cybrids possessed 88% ΔmtDNA4696, while the P29mtP29 and P29mtA11 cybrids did not have any ΔmtDNA4696 (Fig. 1A, lower panel). As expected, no mtDNA was found in parental ρ^0P29 cells (Fig. 1A).

Then, their mitochondrial respiratory function was examined by estimating activities of mitochondrial respiratory complexes (Fig. 1B). Parental ρ^0P29 cells and P29mtΔ cybrids showed overall respiration defects due to complete mtDNA depletion and accumulation of ΔmtDNA4696 possessing a 4,696 bp-deletion mutation, respectively. Specific reduction of complex I activity was exclusively observed in P29mtA11 cybrids, while P29mtP29 cybrids showed normal mitochondrial respiratory function (Fig. 1B).

3-4 Effects of respiration defects on ROS production and nuclear-coded MCL-1 expression

Since we have reported recently [16] that ROS overproduction and resultant up-regulation of nuclear-coded antiapoptotic MCL-1 (myeloid cell leukemia-1), at least in part, are responsible for metastasis, we estimated the amounts of ROS and the nuclear-coded MCL-1 in the cybrids (Fig. 2). The P29mtΔ cybrids did not show ROS overproduction (Fig. 2A) and upregulation of MCL-1 (Fig. 2B). Similar results were obtained in ρ^0P29 cells and the P29mtP29 cybrids (Fig. 2B). On the contrary, the P29mtA11 cybrids expressing complex I defects showed ROS overproduction (Fig. 2A) and upregulation of MCL-1 (Fig. 2B). These observations suggest that ROS overproduction and up-regulation of MCL-1 can not be induced by overall mitochondrial respiration defects or normal respiratory function.

3-5 Effects of mitochondrial respiration defects on glycolytic activity and metastasis

Then, we examined the glycolytic activity of these cybrids and parental ρ^0P29 cells by estimating lactate level in their culture medium. As expected, parental ρ^0P29 cells and P29mtΔ cybrids expressing overall respiration defects (Fig. 1B) showed significant overproduction of lactate (Fig. 3A). Slight overproduction of lactate was observed in P29mtA11 cybrids expressing complex I defects, while P29mtP29 cybrids showed normal lactate secretion (Fig. 3A). These observations suggest that ρ^0P29 cells and P29mtΔ cybrids expressed enhanced glycolysis under normoxia, i.e., the Warburg effect, due to overall mitochondrial respiration defects, but did not express ROS overproduction. Thus, they can be used for determination of whether the Warburg effect alone induces high metastatic potential in tumor cells.

Finally, we examined metastatic potentials by inoculation of the cybrids into the tail vein of male C57BL/6 mice and counting the number of nodules formed in the lung 18 days after inoculation. The ρ^0P29 cells and P29mtΔ cybrids expressing the Warburg effect, but not expressing ROS overproduction did not form lung nodules (Fig. 3B). As expected from our recent report [16], P29mtA11 cybrids expressing the Warburg effect and ROS overproduction formed nodules in the lung, while P29mtP29 cybrids expressing normal respiratory function did not form lung nodules (Fig. 3B). These observations suggest that the mtDNA mutations that induce ROS overproduction can be responsible for metastasis, but the mtDNA mutation or mtDNA depletion that induces the Warburg effect alone are not responsible for metastasis at least in Lewis lung carcinoma cell lines.

4. Discussion

Our recent report demonstrated that complex I defects caused by the mtDNA mutations and resultant ROS overproduction reversibly control metastasis, and that ROS overproduction controls metastasis not by induction of genetic instability or by upregulation of glycolysis, but by upregulation of metastasis-related nuclear genes [16]. However, since complex I defects simultaneously induce upregulation of glycolysis in addition to ROS overproduction, it is still possible that upregulation of glycolysis caused by complex I defects alone can be responsible for metastasis. We examined this possibility by isolation of the P29mtΔ cybrids that expressed enhanced glycolysis, but did not express ROS overproduction (Fig. 2), and showed that metastasis was not induced in the P29mtΔ cybrids (Fig. 3). Since enhanced glycolysis under normoxia, i.e., the Warburg effect, caused by mitochondrial respiration defects alone did not induce

metastasis in the P29mtΔ cybrids, the induction of high metastatic potential in the P29mtA11 cybrids [16] would be due to ROS overproduction, but not to the Warburg effect caused by complex I defects.

The remaining question is whether enhanced glycolysis may induce oncogenic transformation of normal cells to develop tumors, even though it did not induce malignant progression of tumor cells to develop metastasis (Fig. 3B). This possibility would be excluded, since mito-mice expressing enhanced glycolysis due to accumulation ΔmtDNA4696 [23, 24] did not show any bias to form tumors in their tissues (Ishikawa et al., unpublished observations). Thus, enhanced glycolysis induced by mitochondrial respiration defects would not be involved in both tumor and metastasis development at least in tissues of mito-mice and in mouse tumor cells used in this study. To provide direct evidence of whether the Warburg effect induced by mitochondrial respiration defects is not involved in tumor development, we are going to isolate embryonic fibroblast cell lines possessing ΔmtDNA4696 from the mito-mice, and examine whether they are prone to develop tumors.

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system preventing mice from expression of disease phenotypes by mutant mtDNA. Nature Med. 7, 934-940.

Figure legends

Fig. 1 Genotyping of mtDNA and respiratory complex activities.

A: Identification of the mtDNA genotypes. Upper panel: For identification of A11 mtDNA with the G13997A mutation, the PCR products were digested with *Afl* II. The PCR products of the mtDNA with the G13997A mutation from A11 cells produce 114- and 33-bp fragments because of the gain of an *Afl* II site by a G13997A substitution, whereas those of mtDNA without the mutation produce a 147-bp fragment due to the absence of the *Afl* II site. Lower panel: For estimation of ΔmtDNA4696 proportions in the cybrids, we used Southern blot analysis of total DNA digested with the restriction endonuclease *Xho* I. Fragments of 16.3 kbp and 11.6 kbp correspond to wild-type mtDNA and ΔmtDNA4696, respectively. **B**: Biochemical analysis of respiratory complex activities. Respiratory complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), complex III (cytochrome *c* reductase), and complex IV (cytochrome *c* oxidase) are components of electron-transport chain, and are located in the mitochondrial inner membrane. Mitochondrial respiratory function was examined by estimating their activities. Since the activity of complexes II + III is normal in the P29mtA11 cybrids, reduced activity of complexes I + III in them should result from complex I defects. Bars represent the mean ± S.D. ($n = 3$). *, $P < 0.05$.

Fig. 2 Effects of mitochondrial respiratory function on ROS production and MCL-l expression.

A: Estimation of ROS production. We carried out flow-cytometric analysis using 1×10^6 cells. Cells treated with 5 μM MitoSOX-RED were subjected to FACScan for quantitative estimation of superoxide. **B**: Expression of nuclear-coded MCL-1 by Western blot analysis. We used β-actin as loading control in the Western blots.

Fig. 3 Effects of the enhanced lactate production on expression of metastatic potential.

A: The lactate level in culture medium. The P29mtP29 cybrids and ρ0P29 cells were used as cells with normal mitochondrial respiratory function and without mitochondrial respiratory function, respectively. Bars represent the mean ± S.D. (n = 3). *, $P < 0.05$; **, $P < 0.005$. **B**: Experimental metastatic potential. We counted number of nodules formed in the lung after inoculation of the cybrids into the tail vein. Enhanced glycolysis is not necessarily correlated with expression of high metastatic potential. Bars represent the mean ± S.D. (n = 6).