Positive Contribution of Pathogenic Mutations in the Mitochondrial Genome to the Promotion of Cancer by Prevention from Apoptosis

Yuijro Shidara,1,3 Kumi Yamagata,3 Takashi Kanamori,3 Kazutoshi Nakano,2 Jennifer Q. Kwong,4 Giovanni Manfredi,3 Hideaki Oda,1,4 and Shigeo Ohta1

Abstract

The role of mitochondrial dysfunction in cancer has been a subject of great interest and much ongoing investigation. Although most cancer cells harbor somatic mutations in mitochondrial DNA (mtDNA), the question of whether such mutations contribute to the promotion of carcinomas remains unsolved. Here we used trans-mitochondrial hybrids (cybrids) containing a common HeLa nucleus and mtDNA of interest to compare the role of mtDNA against the common nuclear background. We constructed cybrids with or without a homoplasmic pathogenic point mutation at nucleotide position 8,993 or 9,176 in the mtDNA ATP synthase subunit 6 gene (MTATP6) derived from patients with mitochondrial encephalomyopathy. When the cybrids were transplanted into nude mice, the MTATP6 mutations conferred an advantage in the early stage of tumor growth. The mutant cybrids also increased faster than wild type in culture. To complement the mtDNA mutations, we transfected a wild-type nuclear version of MTATP6, whose codons were converted to the universal genetic codes containing a mitochondrial target sequence, into the nucleus of cybrids carrying mutant MTATP6. The restoration of MTATP6 slowed down the growth of tumor in transplantation. Conversely, expression of a mutant nuclear version of MTATP6 in the wild-type cybrids declined respiration and accelerated the tumor growth. These findings showed that the advantage in tumor growth depended upon the MTATP6 function but was not due to secondary nuclear mutations caused by the mutant mitochondria. Because apoptosis occurred less frequently in the mutant versus wild-type cybrids in cultures and tumors, the pathogenic mtDNA mutations seem to promote tumors by preventing apoptosis. (Cancer Res 2005; 65(5): 1655-63)

Introduction

The role of mitochondrial dysfunction in carcinogenesis has been investigated extensively by various approaches. It has been established that the majority of cancer cells harbor homoplasmic somatic mutations in the mitochondrial genome (1–8). However, despite the close association between carcinogenesis and somatic mutations, it remains unclear whether these somatic mutations are contributors to the development of tumors. The high frequency of mitochondrial DNA (mtDNA) mutations and the rapid proliferation of cancer cells with no physiologic advantage may account for the accumulation of somatic neutral mutations in mtDNA. In fact, extensive computer modeling suggests that if a mtDNA mutation occurs in a tumor progenitor cell, mtDNA homoplasy (i.e., a pure population of mutant mtDNA molecules) can be achieved entirely by chance through unbiased mtDNA replication and sorting during cell division without selection for physiologic advantage (9). This model can explain the occurrence of homoplasmic neutral somatic mutations in mtDNA in cancers.

On the other hand, comprehensive scanning of somatic mtDNA has recently revealed that functionally relevant point mutations in mtDNA and polypeptide-encoding genes were detected in 50% of patients (10). Thus, cancer cells seem to harbor pathogenic mutations in mtDNA as well as neutral ones. In addition, a transient depletion of mtDNA causing oxidative phosphorylation dysfunction was found to influence the expression of some nuclear genes involved in tumorigenic and invasive phenotype (11). Furthermore, inherited heterozygous mutations in the nuclear genes encoding for two ubiquitously expressed mitochondrial enzymes, succinate dehydrogenase and fumarate hydratase (fumarase), that catalyze sequential steps in the Krebs cycle, have been shown to cause predisposition to two types of inherited neoplasia syndromes (12–15). These findings prompted us to investigate the relationship between mitochondrial dysfunction and carcinogenesis.

In the present study, we tried to characterize the role of pathogenic mtDNA mutations in the promotion of cancer using two methods. First, we used trans-mitochondrial hybrid cells (cybrids). Cybrids were generated by repopulating HeLa cells devoid of mtDNA with mtDNA derived from enucleated cells of patients harboring heteroplasmic mtDNA mutations in the mitochondrial ATP synthase subunit 6 gene (MTATP6) associated with neuropathy, ataxia, and retinitis pigmentosa (16) or maternally inherited Leigh syndrome (17). This technique allowed for the isolation of cybrid clones containing either homoplasmic mutant or homoplasmic wild-type mtDNA against a common nuclear background (18, 19). Second, we transfected a nuclear version of MTATP6, whose codons were converted to the universal genetic codes containing a mitochondrial localization presequence, into the nucleus of mutant cybrids to complement and restore the defect caused by the mtDNA mutations (20).

Here, we show that two different pathogenic MTATP6 mutations contribute to promotion of tumors by prevention of apoptosis.

Materials and Methods

Isolation and Culture of Cybrid Cell Lines and Their Culture. Primary skin fibroblasts were obtained from a 9-month-old male who had clinical characteristics of maternally inherited Leigh syndrome. His mtDNA
contained a point mutation with a T-to-G transversion at nucleotide position 8,993 in the ATP synthase subunit 6 gene (MTATP6; refs. 16, 17). The fibroblasts were used to construct cybrids as described (19). Platelets were obtained from siblings, an 18-year-old female and a 13-year-old male, who also had clinical characteristics of maternally inherited Leigh syndrome but with a mild clinical form (21). They harbored a T-to-C transition at nucleotide position 9,176 in the MTATP6 gene (21, 22). The enucleated fibroblasts or the platelets were fused with EBV, a β-gal HeLa-derived line that are completely lacking mtDNA and resistant to 8-thioguanine (19). Cybrids were selected with 8-thioguanine and maintained in a glucose-rich medium, DMEM/F-12 (Invitrogen Co., Carlsbad, CA) containing glucose (3.2 mg/mL), pyruvate (0.6 mg/mL), uridine (50 μg/mL), and 10% fetal bovine serum. Mycoplasma contamination was checked monthly.

Detection of Mutant mtDNA. Total DNA from cybrids or tumors was isolated using a DNeasy Tissue Kit (QIAGEN Sciences, Valencia, CA). To detect the T8,993G mutation, a 139-bp PCR fragment was amplified using forward and reverse primers corresponding to mtDNA nucleotide positions 8,917 to 8,935 and 9,043 to 9,055, respectively. PCR products were digested with the restriction enzyme BstYI. The T8,993G mutation creates a novel BstYI site and thus the mutant PCR products are cleaved in 72- and 62-bp fragments. To detect the T9,176C mutation, a 178-bp PCR fragment was amplified using forward and reverse primers corresponding to mtDNA nucleotide positions 9,025 to 9,046 and 9,177 to 9,203 respectively. PCR products were digested with the restriction enzyme ScaFI. The T9,176C mutation creates a novel ScaFI site and thus the mutant PCR products are cleaved in 151- and 28-bp fragments. Digested PCR products were resolved by agarose gel electrophoresis and the relative proportions of mutant and cleaved PCR products were subjected to agarose gel electrophoresis and the relative proportions of mutant and cleaved PCR products were determined using at least six sections from each tumor and cell clone. For each tumor or cell culture, the percentage of positively stained cells was calculated using at least six sections from each tumor and cell clone. For each tumor or cell culture, the percentage of positively stained cells was calculated, and the numbers obtained were averaged to yield the mean ± SD.

For detecting DNA ladders, DNA was isolated from three 2 × 106 cybrid cells (seminconfluent on 100-mm-diameter dishes) using an Apoptotic DNA Ladder Kit (Roche, Indianapolis, IN) according to the manufacturer’s directions. Samples were subjected to agarose gel electrophoresis and stained with ethidium-bromide (24). With this method, DNA from intact nuclei was excluded, and thus the intact DNA did not disturb the pattern of electrophoresis.

To detect apoptotic cells by flow cytometry, cybrid cells were incubated with phenol red-free DMEM/F-12 containing 0.2% charcoal-stripped fetal bovine serum 24 hours before analysis. Trypsinized cells were resuspended in a hypotonic propidium iodide solution (50 μg/mL) containing 0.1% sodium citrate and 0.1% Triton X-100 for 30 minutes, and subjected to flow cytometric analysis with a FACSscan flow cytometer (Becton Dickinson, San Jose, CA; ref. 25). By this method, apoptotic cells were separated from intact cells, regardless of amounts of nuclear DNA.

For judging apoptotic cells, the nuclei of living cells treated with cisplatin were double-stained with propidium iodide and Hoechst 33342 (5 μM/L each). Cells with propidium iodide–positive and fragmented nuclei were judged as dead and apoptotic, respectively.

Statistical Analysis. Differences between two groups were compared using the two-tailed unpaired Student’s t test. P < 0.05 was considered statistically significant.

Results

Characterization of MTATP6 Cybrids. To explore the role of pathogenic mitochondrial mutations on the development of cancer, we constructed cybrids with the β-gal HeLa nucleus and mtDNA with a T-to-G transversion at mtDNA nucleotide position 8,993 (16, 17) or a T-to-C transition at nucleotide 9,176 (21) in the MTATP6 gene. The mutant mtDNA was derived from patients with maternally inherited Leigh syndrome (26). We selected wild-type and mutant homoplasmic cybrid clones (Fig. 1A). Cybrid clones 8W1, 8W2, and 8W3 were homoplasmic wild-type clones; clones 8M7, 8M8, and 8M9 were homoplasmic for the T8,993G mutation; clones 9W4, 9W5, and 9W6 were homoplasmic wild-type clones; clones 9M10 and 9M11 were homoplasmic for the T9,176C mutation. The homoplasmic mtDNA genotypes were confirmed periodically to ensure that there was no drift towards heteroplasmy.

We assessed the respiratory function in the cybrid clones by measuring oxygen consumption rates with an oxygen electrode. As previously described (27), we found that mutant cybrid clones
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had decreased oxygen consumption as compared with wild type (Fig. 1B and C).

Transplantation of Cybrids into Nude Mice. To compare parental ρ0 HeLa, mutant and wild-type cybrids in terms of their potential to form tumors, we injected 5 × 10^6 cybrid cells s.c. into nude mice. Settlement frequencies from the mutant and wild-type cybrids were compared with the 8W1 (wild type) clone. Settlement frequencies from the mutant cybrids were higher than those from the wild type and settlement frequency in mutant and wild-type cybrid clones. When compared the growth rate of tumors (Fig. 2B), the wild-type mtDNA seemed to be selectively excluded in the mixture of the wild-type and mutant cybrids (Fig. 3A and B). Taken together, these findings suggest that mtDNA mutant cells have an apparent advantage in forming tumors as compared with wild-type cells especially in the early stage, providing an explanation for why homoplasmic mtDNA mutations are found in many tumors with mitochondrial dysfunction.

Advantage in Increase of Cultured Mutant Cybrids. To study the faster growth of tumors derived from the mutant cybrids, we compared mutant and wild-type cybrids in terms of their increasing rates in culture. We did growth curve measurements comparing mutant and wild-type cybrids and found that the mutant cybrids increased faster than wild type, especially in the beginning of culture (Fig. 3C). Mutant cybrids started to increase without a lag time, whereas wild types started to increase at day 2. Although we examined the frequency of the Ki-67 positive cells as a proliferation index, no difference among cybrid clones was seen (Supplementary Fig. S2). Then, to confirm the advantage for increasing mutant cybrids, once again, we mixed mutant and wild-type cybrids in a 1:1 ratio and followed the relative proportions of the mutant and wild-type mtDNAs in the mixed cultures over time. This experiment clearly recapitulated our findings in the mixed tumors in the mice, because we observed that the relative content of mutant mtDNA progressively increased and eventually replaced the wild-type mtDNA (Fig. 3D). Because mutant and wild-type cells contained the same amount of mtDNA (see day 0 in Fig. 3D), this in vitro experiment confirmed the advantage for increasing the mutant cybrids.

Expression of Wild Type and Mutant MTATP6 from the Nucleus. It may be possible that mutant mtDNA induces secondary mutations in nuclear genes that accelerate the proliferation of mutant cybrids. This might be one of the molecular mechanisms of how the mutant cybrids gained their apparent advantage in the increase. A direct way to determine the role of mtDNA mutations would be to manipulate the mtDNA. However, a technology to transfect mtDNA into mammalian cells has yet to be established. Thus, alternatively, we transfected in mutant cybrids a nuclear version of MTATP6 whose codons had been converted into universal ones (NuaATP6; ref. 20). The gene contained an NH2-terminal presequence (from the P1 isoform of subunit c of human

![Figure 1. mtDNA genotype and oxygen consumption in HeLa cybrids containing either wild type or mutant mtDNA. A, mtDNA genotyping in cybrid clones. mtDNA amplified from clones derived from a patient with the T8,993G mutation were digested with A/v (lanes 1-3 and 7-9). mtDNA amplified from clones derived from a patient with the T9,176C mutation were digested with ScrFI (lanes 4-6 and 10-11). Lanes 1-11, digested PCR products from cybrid clones 8W1, 8W2, 8W3, 9W4, 9W5, 9W6, 8M7, 8M8, 9M9, 9M10, and 9M11, respectively (agarose gel electrophoresis). Densitometric analyses confirmed that all clones contained either homoplasmic wild type or homoplasmic mutant mtDNA (>98%). B, representative traces of oxygen consumption. Semiconfluent cells of cybrid clones 8M7 and 8W1 were trypsinized and 0.7 to 1.5 × 10^7/mL cells were placed into a respiration chamber (50 μL) equipped with a Clark-type oxygen electrode. The rate of oxygen consumption was slower in the 8M7 (T8,993G mutant) clone as compared with the 8W1 (wild type) clone. C, average rates of oxygen consumption in mutant and wild-type cybrid clones. P < 0.01, 8993W versus 8993M and 9176W versus 9176M as determined by two-tailed unpaired Student’s t test. Columns, averages of three independent experiments; bars, ±SD.](cancerres.aacrjournals.org)
ATP synthase) to target the protein to mitochondria and a FLAG epitope for ease of immunodetection. It was shown that in homoplasmic mutant cybrids this gene product partially restores normal mitochondrial oxidative phosphorylation (20). We isolated stable transfectants with NuATP6 and confirmed the expression of NuATP6 by immunostaining with anti-FLAG antibodies (Supplementary Fig. S3). Conversely, we transfected a nuclear version of MTATP6 containing the T8,993G mutation (muATP6) into wild-type cybrids. As expected, stable transfections of NuATP6 in mutant cybrids partially restored oxygen consumption, whereas introduction of muATP6 into wild-type cybrids decreased oxygen consumption (Fig. 4A). The incomplete rescue of oxygen consumption by NuATP6 in mutant cybrids and the partial decrease by muATP6 in wild-type cybrids are explained by the persistence of endogenous mutant and wild-type MTATP6, respectively.

**Increase Rates of Cybrids Modulated by Transfection with Nuclear Versions of MTATP6.** Increase rates of cybrid transfectants were examined in vitro culture. When NuATP6 was transfected into mutant cybrid 8M7, resulting transfectants (8M7 + NuATP6) grew significantly slower than control mutant cybrids (8M7m) mock transfected with an empty plasmid (Fig. 4B). Conversely, when muATP6 was transfected into 8W1 wild-type

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**Figure 2.** Transplantation of cybrids into nude mice and time course of mtDNA genotype shift during tumor growth. A, settlement efficiency of transplantation. Each cybrids clone (~ 5 x 10⁶ cells) was injected hypodermically into nude mice. Ten days after transplantation, efficiency of tumor formation (% successful tumor implantation for each cybrids clone) was calculated from 12 to 24 experiments. Columns 0 and 1-11, parental ψ₀ cell, cybrid clones 8W1, 8W2, 8W3, 9W4, 9W5, 9W6, 8M7, 8M8, 8M9, 9M10, and 9M11, respectively. B, time course of increase in volume of tumors. Tumors derived from wild type (8W1) versus tumors derived from T8,993G mutant (8M7; P < 0.001 at days 4, 5, and 6; P < 0.01 at day 7; P < 0.05 at days 3, 8, and 9). Tumors derived from the wild type (9W4) versus tumors derived from T9,176C mutant (9M10; P < 0.001 at days 4, 5, and 6; P < 0.01 at day 7; P < 0.05 at days 3, 8, and 9). No significance was found at day 14. Points, average values from three independent experiments; bars, SD. C, representative photographs of 6-day-old tumors derived from clones 8W1 or 8M7 stained with H&E. Bar, 1 mm. Tumor derived from the mutant cybrids seems larger. Top left, portion of magnification. Bar, 10 μm. D, columns, average volumes of the tumors 6 days after transplantation from three independent experiments; bars, SD. P < 0.001, group 8993W versus 8993M and group 9176W versus 9176M. E, average cell number in sections of the maximum surface of tumors 6 days after transplantation from three independent experiments. P < 0.001, group 8993W versus 8993M and group 9176W versus 9176M.
cybrids (8W1 + muATP6), resultant transfectants increased significantly faster than control wild-type cybrids (8W1m) mock transfected with an empty vector. Moreover, to confirm the effect by NuATP6 on the increase of the transfectants, we mixed 8M7 + NuATP6 and mock transfected 8M7m in a 1:1 ratio. We also measured the increase rates of 8W1m mock and muATP6 transfected cybrids mixed in a 1:1 ratio. Because both muATP6 and NuATP6 were tagged with FLAG, we were able to identify transfectant cells throughout the duration of the experiment. After 6 days, there was a decrease in the number of NuATP6 FLAG-positive cybrids relative to mock transfected (Fig. 4C and Supplementary Fig. S4, left). Conversely, there was an increase in the number of muATP6 FLAG-positive cybrids as compared with the mock transfectant in the mixed cultures (Fig. 4C and Supplementary Fig. S4, right). These findings indicate that the modulation of mitochondrial ATP synthase activity via expression of mutant or wild-type MTATP6 from the nucleus can affect cell proliferation and override the effect of the mtDNA genotype.

Next, we transplanted the 8M7 + NuATP6 or 8W1 + muATP6 cybrids into nude mice and followed tumor growth. The results indicated that the expression of a functional MTATP6 in 8M7 + NuATP6 cybrids slowed down tumor growth as compared with tumors derived from mock transfected 8M7m; whereas a defective MTATP6 in 8W1 + muATP6 cybrids promoted tumor growth as compared with tumors derived from mock transfected 8W1m cybrids (Fig. 4D).

To recapitulate in vivo the growth advantage conferred by muATP6 to wild-type cybrids, we mixed in a 1:1 ratio 8M7 + NuATP6 cybrids with mock 8M7m ones, or conversely, 8W1 + muATP6 cybrids with mock 8W1m, and transplanted the mixtures into nude mice. Confirming the results in cell culture, FLAG-positive mutant cybrids expressing NuATP6 resulted in smaller tumors, indicating that functional MTATP6 conferred a disadvantage in tumor growth (Supplementary Fig. S5, left) as compared with expression of defective MTATP6 (Supplementary Fig. S5, right). A quantitative analysis of FLAG-positive cybrids using several transfected cybrid clones confirmed the reproducibility of these observations in forming tumors derived from cybrids (Fig. 4E). Taken together, these observations consistently showed that the increase advantage in culture and the growth advantage in vivo tumor depends upon the decline in MTATP6 function, and not upon secondary effects from the nucleus.

Lower Frequency of Apoptosis of Mutant Cybrids In vitro and In vivo. To explore the molecular mechanism underlying the growth advantage in tumors, we hypothesized that the mutant mtDNA may protect cells from apoptosis, and therefore growth advantage of mutant cybrids may derive from increased survival. To test this hypothesis, apoptosis was examined by three independent methods. First, we looked at TUNEL staining during the course of 8 days of continuous culture. Although TUNEL-positive cells were rare, they were more abundant in wild-type cybrids than in mutant cybrids throughout the duration of culture (Fig. 5A and Supplementary Fig. S6). Especially at day 1, TUNEL-positive cells were more in wild-type cybrids, which agrees with the finding of the lag time as seen in Fig. 3C. Second, DNA fragmentation was more extensive in the wild type than in the mutant cybrids (Fig. 5B). Third, flow cytometric analysis revealed that the population of apoptotic cells belonging to sub-G1 phase was more abundant in the mutant (~ 8% of total cells) than in the wild-type cybrids (~ 2% of total cells; Fig. 5C and D). Thus, all three methods confirmed that wild-type cybrids undergo spontaneous apoptosis more frequently than mutants. In addition,
profiles for the cell size (data not shown) and the cell cycle obtained by flow cytometry did not markedly differ between the mutant and wild-type cybrids (Fig. 5C), which agrees with the Ki-67 staining as seen in Supplementary Fig. S2. Then, we compared sensitivities to apoptosis when cells were exposed against an apoptosis-inducing stimulus. When cybrids were treated with cisplatin, an apoptosis inducer, mutant cybrids presented less dead (propidium iodide positive) and apoptotic with fragmented nuclei as judged by Figure 4.

Transfection of cybrids with nuclear versions of the ATP synthase subunit 6 gene and the growth characteristics of the transfectants. Cybrids were transfected with FLAG tagged nuclear versions of the ATP synthase subunit 6 gene, NuATP6 (wild type) or muATP6 (mutant). A, columns, average values of oxygen consumption in transfected cybrids obtained from three independent experiments: 8M7m, 8W1m, 9M10m, and 9W5m are mock transfectants with an empty plasmid of cybrid clones 8M7, 8W1, 9M10, and 9W5, respectively. (8M7 + NuATP6), (9M10 + NuATP6), and (8W1 + muATP6) are transfusions with NuATP6 of 8M7, 9M10, and 8W1 cybrids, respectively. P < 0.01, 8M7m versus (8M7 + NuATP6), 8W1m versus (7 + NuATP6) and each other mock transfectant versus each corresponding transfectant with NuATP6 or muATP6. B, growth curves. 8W1m and 8M7m are control cybrids mock (m) transfected with an empty vector, whereas (8M7 + NuATP6) and (8W1 + muATP6) are cybrids stably expressing the NuATP6 and muATP6, respectively. P < 0.01, mock transfected controls versus transfectants with NuATP6 and mock transfected controls versus transfectants with muATP6 on each day of culture in three independent experiments. C, mixture at a ratio of 1:1 of (8M7 + NuATP6) and 8M7m or (8W1 + muATP6) and 8W1m was cultured for 6 days and immunostained with anti-FLAG antibodies. Proportion of FLAG-positive cells (expressed as % total cells) in mixed cultures on day 6. Columns 8M7, 8M8, or 8M9, proportion of FLAG-positive cells in the mixture of (8M7 + NuATP6) and 8M7m, (8M8a + NuATP6) and 8M8m, or (8M9 + NuATP6) and 8M9m, respectively. Columns 8W1, 8W2, or 8W3, proportion of FLAG-positive cells in the mixture of (8W1 + muATP6) and 8W1m, (8W2 + muATP6) and 8W2m, or (8W3 + muATP6) and 8W3m, respectively. Columns, averages of three independent experiments; bars, ± SD. P < 0.001, group 8M versus group 8W. D, time course of tumor growth. Transfected cybrids were transplanted into nude mice and tumor size was measured at the time points indicated. The average sizes of tumors derived from 8M7 expressing NuATP6 (8M7+NuATP6) as compared with 8M7 transfected with empty vector (8M7m), and of 8W1 cybrids expressing muATP6 (8W1+muATP6) as compared with 8W1 cybrids transfected with empty vector (8W1m) were both significant (P < 0.001). Points, average values from three independent experiments; bars, SD. E, proportions of transfectants expressing FLAG on day 6 after transplantation into nude mice. 8M7m and 8M7+NuATP6 or 8W1m and 8W1+muATP6 were mixed 1:1 and transplanted into nude mice. After 6 days, tumors were fixed and immunostained with anti-FLAG antibodies and FLAG-positive and FLAG-negative cells were counted. Average proportions of FLAG-positive were obtained from three independent experiments. 8M7, proportion of FLAG-positive cells in a 1:1 mixture of 8M7m and 8M7+NuATP6. 8W1, proportion of FLAG-positive cells in a 1:1 mixture of 8W1m and 8W1+muATP6. Columns, averages of three independent experiments; bars, SD.
morphology (Fig. 5E and F). Thus, the mutant cybrids are more resistant against apoptosis than the wild type.

Finally, we compared the frequency of apoptosis and proliferation rate in tumors derived from cybrid transplantation. We counted the number of Ki-67- and TUNEL-positive cells in tumors. Although TUNEL-positive cells were rare, they were significantly more numerous in tumors derived from wild type than from mutant cybrids (Fig. 6A and Supplementary Fig. S7A). On the other hand, there was no marked difference in Ki-67 positive cells (a proliferation marker; 28) between tumors (Fig. 6B and Supplementary Fig. S7B). These results indicate that the mutant mtDNAs suppress apoptosis in tumors as well as in cultured cybrids.

Discussion

Mitochondrial defects have long been suspected to play an important role in the development of cancer. Fifty years ago, Warburg pioneered the research on the involvement of mitochondrial respiratory defects in cancer, and proposed a mechanism to explain how these defects evolve during carcinogenesis. Warburg hypothesized that a key event in carcinogenesis involved the development of an injury to the mitochondrial respiratory machinery, resulting in a compensatory increase in glycolysis, leading to lactic acidosis (29). Lactic acidosis is also a typical biochemical hallmark of mitochondrial diseases and it is widely used in the diagnosis of mitochondrial encephalomyopathies (26, 30).

Although it has been shown that a majority of cancer cell lines harbor mutant mtDNA, it has not yet been determined whether mtDNA mutations precede and lead to carcinogenesis. In light of Warburg’s theory, it would be especially interesting to better understand the role of mutant mtDNA associated with dysfunction of mitochondria in carcinogenesis. Furthermore, the high frequency of mtDNA alterations in cancer and their presence in the early stages of disease could perhaps be exploited as clinical markers for early cancer detection (3, 31–33). The mtDNA alterations detected in cerebrospinal fluid may be used as sensitive markers to monitor disease progression and predict relapse (34).

The transplantation of cybrids into nude mice has been employed previously to investigate the role of mtDNA in tumorigenesis. Hayashi et al. (35) transplanted cybrid cells and \( \rho^0 \) cells lacking mtDNA into nude mice and reported that tumors formed by the cybrids containing wild-type mtDNA but not by \( \rho^0 \) cells devoid of mtDNA. In contrast, Morais et al. (36) reported that \( \rho^0 \) cells could form tumor. Because HeLa \( \rho^0 \) cells did form tumors but at a very low frequency in our study, the potential for forming tumors may depend upon the viability of \( \rho^0 \) cells. In their experiments, they have not compared cybrids with wild type or mutant mtDNA. This is a very relevant issue because mtDNA
mutations arise spontaneously in somatic cells at a relatively high rate. Thus, the potential role of mtDNA mutations in cancer development, genetic instability, and disease progression warrants a careful and comprehensive investigation.

In the present study, as a model, we used cybrids harboring mtDNA with or without pathogenic mutations at nucleotide positions 8,993 or 9,176 in the MTATP6 gene. This cybrid system has an advantage that the mtDNA sequences are identical except for a single pathogenic mutation between mutant and wild-type clones, because both the mutant and wild-type mtDNAs are derived from the same subject (37). In contrast, cancer cells tend to have multiple mutations in mtDNA (38) making it difficult to correlate any phenotypic differences with a specific mutation. We chose to use cybrids harboring the MTATP6 gene mutations for two reasons. First, cancer cells often contain mutations in the MTATP6 gene (6–8) and, in addition, most renal carcinomas lose ATP synthase activity (39). Second, we took an advantage of the possibility of manipulating the content of mutant or wild-type MTATP6 in cells by a previously established technique of nuclear gene transfer of MTATP6 (20). The ability to manipulate the growth phenotypes of cancer cells expressing exogenous MTATP6 allowed us to confirm that these phenotypes were directly due to mtDNA mutations and not to secondary nuclear modifications. However, this study does not despise the predominant role of the nucleus (35) and does not exclude the possibility that mutant mtDNA may influence the expression of genes involved in cell proliferation, for example by affecting the mitochondrial regulation of intracellular Ca\(^{2+}\) levels (40).

Our study shows that cybrids harboring mutant mtDNA increase faster in culture and are more efficient at promoting tumorigenesis, as compared with cybrids harboring wild-type mtDNA, especially in the early stage when no marked massive necrotic cell death was seen. In addition, the concept that mutant mtDNA can overcome wild-type mtDNA within one cell is suggested by previous experiments where cells with and without mutant mtDNA were fused to each other (2). Furthermore, it has been shown that the proportion of certain pathogenic mutant mtDNAs tends to expand over time in muscle or brain of patients with mitochondrial encephalomyopathy (41). Thus, it is no surprise that mtDNA molecules harboring certain somatic mutations may quickly become the dominant species or even become entirely homoplasmic in cancer cells.

The molecular mechanism by which mutants faster than wild-type cybrids remains to be fully clarified. Because we could see no marked difference in the proliferation index of the Ki-67 antigen \textit{in vitro} and \textit{in vivo}, one potentially important information that emerged from our studies, which may explain the different growth between mutant and wild-type cybrids, is the extent of apoptosis. Because apoptosis plays a critical role in cancer development and in the cellular response to anticancer agents (42, 43), the effect of mtDNA mutations on apoptosis in cancer cells is obviously an important area of investigation. The effect of mtDNA mutations on apoptosis is still unclear. In some experiments, \(\rho^0\) cells lacking mtDNA have been used as a model for defective mtDNA. For instance, it was reported that \(\rho^0\) cells were more resistant to a reagent that induces apoptosis than parental cells (44). On the other hand, \(\rho^+\) cells were shown to be more sensitive to apoptosis than their \(\rho^+\) parental cells in culture (45) and \textit{in vivo} (46). One study showed that cytochrome \(c\) release and caspase activation in response to staurosporine treatment were maintained in \(\rho^0\) cells (47). However, few experiments have compared cybrids with and without mutant mtDNA. Therefore, the exact role of pathogenic mtDNA mutations in the cellular apoptotic response remains to be defined.

Although our study suggests that loss of apoptosis may have an important role in fostering growth in tumors derived from mutant cybrids, it is not clear whether the difference in the frequency of apoptosis can fully explain the difference in tumor growth or growth rate in culture. The frequency of TUNEL-positive cells in tumors from mutant cybrids was rather small, but because TUNEL-positive apoptotic cells seem transiently and continuously during the life of the tumor, dead cells may should be more than the TUNEL-positive cells. Thus, estimating the frequency of apoptotic cell death in tumors just by TUNEL staining may lead to underestimation. In fact, a flow cytometric analysis of apoptotic features in cultured cells revealed a greater proportion of apoptotic cell death than TUNEL staining. Additionally, because wild-type mtDNA markedly decreased in the genetic drift experiments using the mixture of wild-type and mutant cybrids, the wild-type cybrids themselves seemed to be eliminated (Fig. 3A, B, and D). Moreover, wild-type cybrids in culture began to increase with a lag time accompanying apoptosis (Figs. 3C and 5A). Because settlement frequencies from wild-type cybrids were lower than those from mutants, the early events may be crucial in the settlement and promotion of tumorigenesis (Fig. 24).
Our findings seem to be consistent with a report that (48) fibroblasts harboring the 8,993 MTATP6 mutation were more sensitive to metabolic stress and apoptosis than wild-type fibroblasts. However, this discrepancy is likely to be due to the difference between primary fibroblasts and cancer cells such as HeLa cybrids, for example, the glycolytic pathway in HeLa cells is more active than that in fibroblasts. Here we presented positive contributions of two pathogenic mutations of mtDNA to the promotion of tumorigenesis. However, it will be required to use mutant mtDNAs derived from actual cancer tissues instead of mtDNA from patients with mitochondrial encephalomyopathy. Because the physiologic advantage of two pathogenic mtDNAs in tumorigenesis was revealed for the first time, investigations on mtDNA will be more important in conquering cancers.

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9. Coler KA, Khrapko K, Bodyak ND, Nikhawa E, Pfeiffer K. Sensitivity of human cells lacking mtDNA to heteroplasmic and homoplasmic mtDNA mutations in human tissues instead of mtDNA from patients with mitochondrial encephalomyopathy. Because the physiologic advantage of two pathogenic mtDNAs in tumorigenesis was revealed for the first time, investigations on mtDNA will be more important in conquering cancers.

Our findings seem to be consistent with a report that (48) fibroblasts harboring the 8,993 MTATP6 mutation were more sensitive to metabolic stress and apoptosis than wild-type fibroblasts. However, this discrepancy is likely to be due to the difference between primary fibroblasts and cancer cells such as HeLa cybrids, for example, the glycolytic pathway in HeLa cells is more active than that in fibroblasts. Here we presented positive contributions of two pathogenic mutations of mtDNA to the promotion of tumorigenesis. However, it will be required to use mutant mtDNAs derived from actual cancer tissues instead of mtDNA from patients with mitochondrial encephalomyopathy. Because the physiologic advantage of two pathogenic mtDNAs in tumorigenesis was revealed for the first time, investigations on mtDNA will be more important in conquering cancers.

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Positive Contribution of Pathogenic Mutations in the Mitochondrial Genome to the Promotion of Cancer by Prevention from Apoptosis

Yujiro Shidara, Kumi Yamagata, Takashi Kanamori, et al.


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