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REVIEW

Contribution of somatic mutations in the mitochondrial genome to the development of cancer and tolerance against anticancer drugs

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Mitochondrial defects have long been suspected to play an important role in the development of cancer. Although most cancer cells harbor somatic mutations in mitochondrial DNA (mtDNA), the question of whether such mutations positively contribute to the development of cancer remained unclear. To clarify the role of mutant mtDNA excluding effects by the nuclear background, we focus on a method of transmitochondrial cybrids. Tumors were formed by transplanting cybrids with or without mutant mtDNA into nude mice and compared each size, revealing that mutant cybrids enhanced tumorigenesis. Next, we discuss a method for excluding the possibility of secondary nuclear mutations that may affect tumorigenesis. Mitochondrial genes that had been converted from mitochondrial to nuclear codons and equipped with a mitochondrial-targeting sequence were introduced into the nucleus of mutant cybrids. The gene products complemented the dysfunction, and reduced the promotion of tumors. By these methods, we concluded that mutant mitochondria positively and directly contribute to tumorigenesis. Since apoptosis occurred less frequently in the mutant versus wild-type cybrids in tumors, pathogenic mtDNA mutations contribute to the promotion of tumors by preventing apoptosis. Finally, we discuss the role of mutant mtDNA in conferring tolerance against anticancer drugs.
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Introduction

The role of mitochondrial dysfunction in cancer has been a subject of great interest from various aspects. Mitochondrial defects have long been suspected to play an important role in the development of cancer. Over 50 years ago, Warburg pioneered research into the involve-

ment 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 (Warburg, 1930, 1956). Lactic acidosis is a typical biochemical hallmark of mitochondrial diseases and is widely used in the diagnosis of mitochondrial encephalomyopathies (Di-Mauro *et al.*, 1985). Many groups have confirmed that the majority of cancer cells harbor somatic mutations in the mitochondrial genome which may represent the 'injury of mitochondria' described by Warburg (Polyak *et al.*, 1998; Yeh *et al.*, 2000; Liu *et al.*, 2001; Parrella *et al.*, 2001; Carew and Huang, 2002; Maximo *et al.*, 2002; Tan *et al.*, 2002). Comprehensive scanning of somatic mitochondrial DNA (mtDNA) mutations has revealed that functionally relevant point mutations in mitochondrial RNA and polypeptide-encoding genes were present in 50% of patients (Linnartz *et al.*, 2004). Thus, cancer cells seem to harbor pathogenic mutations in mtDNA as well as neutral mutations.

However, despite the close association between carcinogenesis and somatic mutations, it remained unclear whether these somatic mutations are contributors to the development of tumors or whether they simply arise as part of the secondary effects in cancer. In any case, a high frequency of mtDNA alterations in cancer and their presence in the early stages could be exploited as clinical markers for early cancer detection (Fliss *et al.*, 2000; Nishikawa *et al.*, 2001). Mitochondrial DNA alterations detected in cerebrospinal fluid may be used as sensitive markers to monitor disease progression and predict relapse (Wong *et al.*, 2003).

The high frequency of mtDNA mutations and the rapid proliferation of cancer cells with no physiological advantage has been proposed to account for the accumulation of somatic mutations in mtDNA. In fact, extensive computer modeling suggests that if a single mtDNA mutation occurs in a tumor progenitor cell, mtDNA homoplasmy (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 physiological advantage (Coller *et al.*, 2001). This model can explain the occurrence of neutral mutations in cancer mtDNA.

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In this review article, we will discuss a method developed to clarify the role of pathogenic mutations in mtDNA through the investigation of mitochondrial diseases. Here, we applied this method to study the contribution of mutant mtDNA in cancer by focusing on mtDNA and concluded that pathogenic mutations contribute to the promotion of tumorigenesis. Moreover, the association between mtDNA mutations and development of anticancer drug tolerance will also be addressed.

Methods that clarify contributions of mitochondrial DNA

How to investigate the role of mitochondrial DNA

The cell contains a nuclear genome and a mitochondrial genome. The human mitochondrial genome encodes two ribosomal RNA, 22 transfer RNA and 13 polypeptides. All the polypeptides are subunits belonging to the respiratory chain or the ATP synthase complex. The remaining hundreds of mitochondrial proteins are encoded by nuclear genes, synthesized in cytosol and post-translationally imported into mitochondria (Attardi and Schatz, 1988). All components involved in the maintenance, expression and regulation of the mitochondrial genetic system are encoded in nuclear genes. Thus, even though mitochondrial alterations have been described, the role of the mitochondrial genome in promoting malignant transformation is not understood. Additionally, cross-talk between these two genomes should be considered (Ohta, 2003; Nakashima-Kamimura et al., 2005). Thus, when investigating the role of the mitochondrial genome, we must precisely distinguish the contribution of the mitochondrial genome from that of the nuclear genome. In particular, it would be important to understand whether many of these mutations occur during the initiation and/or development of cancer. When approaching this question, it is important to compare mtDNA in the context of identical nuclear backgrounds.

Previously, we developed a method to exclude the contribution of the nucleus to clarify the role of mutant mtDNA derived from patients with mitochondrial disorders. We constructed a hybrid cell with a common nucleus and mtDNA of interest derived from patients and could clarify that several mutations in mtDNA are conclusively responsible for the diseases. Since the cytoplasm containing mtDNA was fused with a cell lacking mtDNA, we call it a 'cybrid' instead of a 'hybrid' (Hayashi et al., 1991a; Attardi et al., 1995).

Establishment of cells lacking mitochondrial DNA

Before referring to cybrids, we will explain the method to completely deplete mtDNA. Since human mtDNA encodes only 13 subunits belonging to the respiratory chain or the ATP synthase complex, mammalian cells can survive without mitochondria if essential metabolic substrates are supplied. ATP can be synthesized in cytosol by glycolysis. In addition to energy metabolism, mitochondria have many other roles that are essential

for living cells. Thus, mitochondria are essential for eukaryotes and cannot be excluded. However, it is possible to deplete mtDNA in cells. Indeed, yeast strains (*Saccharomyces cervisiae*) lacking mtDNA, termed $\rho 0$ are easily established when supplying fermentable carbon sources. However, yeast mitochondria could not be removed. Subsequently removal of mtDNA was also achieved in avian cells (Desjardins et al., 1986). Human $\rho 0$ cells could be isolated by long exposure to ethidium bromide (EtBr), which is a strong inhibitor of mitochondrial RNA polymerase (King and Attardi, 1989: Hayashi et al., 1991b). Since RNA functions as a primer for mitochondrial DNA replication, EtBr strongly inhibits DNA replication. For a long time, mouse $\rho 0$ cells could not be isolated because they became resistant to EtBr at a high frequency (Hayashi et al., 1990). Instead they were isolated by long exposure with an anticancer drug (Inoue et al., 1997). Human $\rho 0$ cells are cultured in a medium containing pyruvate and uridine because pyruvate is converted to lactate coupled with an oxidation of NADH to NAD^+, which is required for glycolysis and because uridine synthesis requires energized mitochondrion (King and Attardi, 1989). Currently, $\rho 0$ cells are often used as a model of a mtDNA mutation, but one should remember that it is highly possible to introduce considerable mutations into nuclear genes of $\rho 0$ cells by long exposure to EtBr. Thus, their parental cells are unsuitable for $\rho 0$ cell control.

No method for the direct introduction of mitochondrial DNA

So far, no technique to introduce full length or a fragment of mtDNA into mammalian cells has been established. Such an approach could enable to introduce a mutation of interest into a specific cancer cell to clarify the role of mutant mtDNA. On the other hand, it is possible to introduce a fragment of yeast mtDNA into yeast with a particle gun. Namely, metal particles with a fragment of mtDNA are shot mechanically into mitochondria across the plasma, and mitochondrial outer and inner membranes at high speed (Klein and Fitzpatrick-McElligott, 1993). Since yeast mitochondria have a strong recombination system, the fragment is inserted into yeast mtDNA by recombination (D'Aurelio et al., 2004). However, human mitochondria have very low mtDNA recombination ability. In addition, mammalian mtDNA is easily degraded if not enveloped by mitochondrial transcriptional factor A (Tfam) (Takamatsu et al., 2002); hence it is difficult to overcome the problems. A revolutionary method is yet to be established for manipulating mtDNA in human cells.

Construction of cybrids

A cybrid is constructed by fusing cytoplasm carrying mtDNA of interest with a $\rho 0$ cell (Figure 1) (Moraes et al., 2001). Cytoplasm is prepared from enucleated cells (Hayashi et al., 1991a); alternatively platelets or synaptosomes can be used as a cytoplasmic source (Inoue et al., 1997). Since a chemical enucleating method by the exposure to actinomycin D has recently been
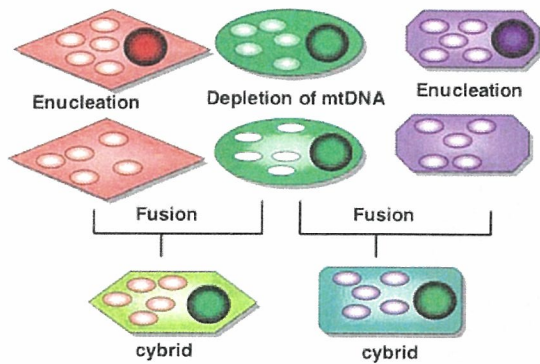
Figure 1 Construction of cybrids. Mitochondrial DNA (mtDNA) is depleted from a nucleus donor by exposure to ethidium bromide (EtBr). On the other hand, cytoplasm carrying the mtDNA of interest is prepared by removing the nucleus by treatment with cytocharasin B followed by centrifugation. Alternatively, platelets or synaptosomes can be substituted with the cytoplasm (Inoue et al., 1997) or a cell treated with actinomycin D is available as a source of mtDNA (Bayona-Bafaluy et al., 2003). The cytoplasm and $\rho 0$ cells were fused to construct cybrids and the resultant cybrids have a common nucleus and differ in mtDNA.

Figure 2 Tumors formed by the transplantation of mutant and wild-type cybrids. Each cybrid clone (approximately 5×10^6 cells) was injected hypodermically into nude mice to form tumors. Representative photographs of 10-day-old tumors derived from clones possessing mutant mitochondrial DNA (mtDNA) at nucleotide position 8993 (left panel) and wild-type mtDNA (right panel). Tumors derived from mutant and wild-type cybrids were stained with hematoxylin and eosin. Scale bar: 1 mm.

reported for the transfer of mtDNA to $\rho 0$ cells (Bayona-Bafaluy et al., 2003), it has become easier to prepare sources of mitochondria from cancer cells.

Cybrids enabled us to confirm that mutations in mtDNA are necessarily and sufficiently responsible for decreases in mitochondrial activity. A deletion mutation was shown to be responsible for the decline in chronic progressive external ophthalmoplegia (CPEO) subgroup mitochondria (Hayashi et al., 1991a). A point mutation in the tRNA^{Lys} gene at nucleotide position 8344 was confirmed to be responsible for the other subgroup myoclonic epilepsy associated with ragged-red fibers (MERRF) (Chomyn et al., 1991). A point mutation in the tRNA$^{Leu(UUR)}$ gene at nucleotide position 3243 or 3271 was confirmed as the cause of the mitochondrial encephalopathy, lactic acidosis and stroke-like episodes (MELAS) subgroup of mitochondrial disease (King et al., 1992; Hayashi et al., 1993). The cybrid method is at present widely used to determine whether a nucleotide substitution is pathogenic or the result of a simple polymorphism.

Direct contribution of mutants mitochondrial DNA revealed by the cybrid method

Transplantation of cybrids into nude mice

To explore the role of pathogenic mitochondrial mutations in the development of cancer, we applied the cybrid method with a HeLa nucleus and mtDNA mutated at nucleotide position 8993 (Holt et al., 1990; Tatuch et al., 1992) or position 9176 (Nakano et al., 2003) in the subunit 6 gene of ATP synthase (MATP6). These mutants are derived from patients with mitochondrial encephalomyopathy. However, they can be reasonably used as models of cancer mtDNA because of the
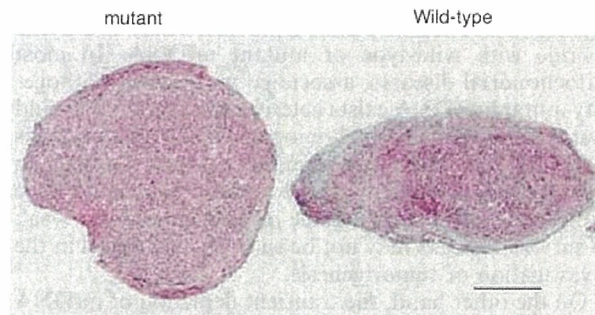
reported downregulation of ATP synthase β-subunit expression in liver, kidney, colon, squamous oesophageal and lung carcinomas, as well as in breast and gastric adenocarcinomas (Cuezva et al., 2002, 2004; Isidoro et al., 2004). To compare mutant and wild-type cybrids in terms of their potential to form tumors, we transplanted the cybrid cells subcutaneously into nude mice. Settlement frequencies from the mutant cybrids were higher than those from the wild type. All the tumors derived from mutant cybrids were larger and grew faster than those from wild-type cybrids (Figure 2) (Shidara et al., 2005). At the same time, the other group reported that cybrids with the same 8993 mutation grew to larger tumors in the same transplantation system (Petros et al., 2005). We would like to emphasize that two independent groups confirmed this result by the cybrid method.

Moreover, to confirm that mutant cybrid tumors had faster growth, we transplanted a mixture (1:1) of mutant and wild-type cybrids into nude mice. The proportion of mutant mtDNA in the tumor increased progressively, and eventually, mutant mtDNA entirely replaced the wild-type mtDNA. Thus, wild-type mtDNA appeared to be selectively excluded from the mixture of wild-type and mutant cybrids. Taken together, mtDNA mutant cells have an apparent advantage to form tumors as compared to wild-type cells, providing an explanation for why homoplasmic mtDNA mutations are found in many tumors with mitochondrial dysfunction (Shidara et al., 2005).

Use of cells lacking mitochondrial DNA

The transplantation of cybrids into nude mice has been employed previously to investigate the role of mtDNA in tumorigenesis. $\rho 0$ cells and cybrid cells with wild-type mtDNA were transplanted into nude mice. Tumors were formed by the cybrids containing wild-type mtDNA but not by $\rho 0$ cells (Hayashi et al., 1992). In contrast, another group reported that $\rho 0$ cells could form tumors

(Morais *et al.*, 1994). However, neither study compared cybrids with wild-type or mutant mtDNA. In most mitochondrial diseases, associated with severe pathogenity mutant mtDNA exists heteroplasmically, while mild mutant mtDNA exists homoplasmically (Lightowlers *et al.*, 1997). Since most somatic mtDNA mutants in cancer reach homoplasmy, the mutations seem to have rather mild pathogenity. Since $\rho 0$ cells may be too weak to survive, $\rho 0$ cells may not be suitable as a model in the investigation of tumorigenesis.

On the other hand, the transient depletion of mtDNA influenced the expression of various nuclear genes involved in tumorigenic and invasive phenotypes (Amuthan *et al.*, 2001).

How to distinguish direct effects by mutant mitochondrial DNA from secondary nuclear mutations

Possibility of secondary mutations in nuclear genes
Since nuclear mutations as well as mtDNA mutations arise somatically at a high frequency in cancer, the potential role of mtDNA mutations in cancer development, genetic instability, and disease progression requires a careful and comprehensive investigation. Secondary mutations in nuclear genes may occur to enhance carcinogenesis during culture or transplantation after the construction of cybrids. If so, it cannot be concluded that mtDNA directly contributes to tumorigenesis, and mutant mtDNA may simply play a role as a mutagenesis of nuclear genes. Mutant mtDNA enhances the generation of reactive oxygen species (ROS), from the mitochondrial electron transport chain, that induces mutations in nuclear genes as well as mitochondrial genes. To address this issue, it would be useful to replace a mutation of mtDNA into wild type and then to examine whether potential effects on tumorigenesis are lost or remain in cybrids. However, as mentioned above, a method for manipulating mtDNA is not available. Instead of manipulating mtDNA, the import of mitochondrial proteins from cytosol into mitochondria was considered to complement mitochondrial dysfunction.

Import of mitochondrial proteins
Most mitochondrial proteins are encoded by nuclear genes, synthesized in cytosol as precursors and imported into mitochondria. Most precursor proteins are equipped with a target presequence at their N-terminus. Even if passenger proteins are not mitochondrial proteins, most of them can be imported into mitochondria when fused with a target presequence (Attardi and Schatz, 1988).

Mitochondrial genes encoding proteins are composed of mitochondrial codons. Thus, at first, codons of mitochondrial genes were converted into universal codons, and genes equipped with a target sequence gene were introduced into the nucleus and expressed in cytosol. The mitochondrial protein produced was imported into mitochondria and complemented the
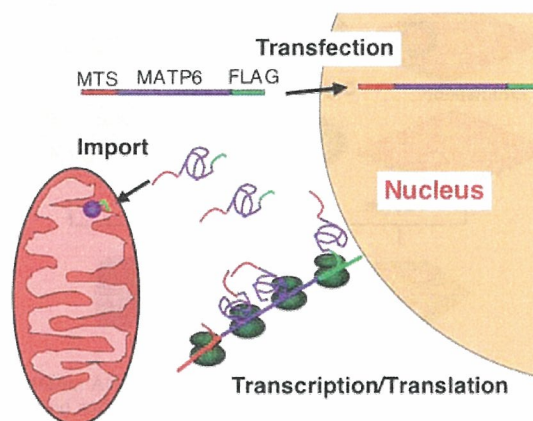

Figure 3 Expression of the mitochondrial ATP synthase subunit 6 (*MATP6*) nuclear transgene in the cytosol and its import into the mitochondria. The *MATP6* gene was converted to the nuclear version by substituting mitochondrial codons with universal codons, fused with a mitochondrial-targeting presequnce (MTS) and a FLAG tag (FLAG) for detection. The gene, wild type or mutant, was transfected into the nucleus and its product functioned in mitochondria by importing from cytosol.

dysfunction derived from the mitochondrial disease (Figure 3) (Manfredi *et al.*, 2002). Additionally, the subunit 4 gene of complex I (*ND4*), a mitochondrial gene, complemented the dysfunction (Guy *et al.*, 2002). It is unknown how universally applicable this method is. Larger and/or more hydrophobic proteins may be difficult to import into mitochondria even when a target presequence is equipped to the protein.

Expression of a mitochondrial protein from the nucleus via cytosol
We explored whether mutant mtDNA induced secondary mutations in nuclear genes that accelerate the proliferation of mutant cybrids. As mentioned above, technology to transfect mtDNA into mammalian cells has yet to be established. Alternatively, we transfected into mutant cybrids a nuclear version of *MTATP6* whose codons had been converted into universal codons (*NuATP6*) (Shidara *et al.*, 2005). The gene contained an N-terminal presequence to target the protein to mitochondria and a FLAG tag for ease of immunodetection. We isolated stable transfectants with *NuATP6* and confirmed the expression of NuATP6 by immunostaining with anti-FLAG antibodies in cybrids carrying the mitochondrial T8993G mutation. Conversely, we transfected a nuclear version of *MTATP6* possessing the T8993G mutation (*muATP6*) into wild-type cybrids. As expected, stable transfections of *NuATP6* in mutant cybrids partially restored oxygen consumption, whereas the introduction of *muATP6* into wild-type cybrids decreased oxygen consumption. The cybrid transfectants were transplanted into nude mice and followed the tumor growth. The results indicated that the expression of functional MTATP6 slowed down tumor growth as compared to tumors derived from mock-transfected cybrids; whereas defective MTATP6 cybrids promoted

tumor growth as compared to tumors derived from mock-transfected cybrids. FLAG-positive mutant cybrids expressing wild-type MATP6 resulted in smaller tumors, indicating that functional MTATP6 conferred a disadvantage in tumor growth as compared with the expression of defective MTATP6. These results clearly show that tumorigenicity depends upon the mitochondrial function, but not upon secondary mutations in the nuclear genome.

To recapitulate *in vivo* the growth advantage conferred by muATP6 compared to wild-type cybrids, we mixed 1:1 ratio cybrid transfectants having functional MATP6 with mock ones, or conversely, cybrid transfectants having mutant MATP6 with mock transfectants, and transplanted the mixtures into nude mice. Since transfection genes were equipped with a FLAG tag, the transfectants were easily detected. Cybrids possessing mutant MATP6-FLAG became numerous in tumors. Conversely, T8,993G mutant cybrids with wild-type MATP6-FLAG were reduced in tumor promotion.

Taken together, these observations consistently showed that the growth advantage of *in vivo* tumors depends on the decline in the MATP6 function, and not upon secondary effects from the nucleus.

How to increase mutant mitochondrial DNA after a somatic mutation

Dual roles of reactive oxygen species in the development and prevention of cancer
As shown above, pathogenic mutations in mtDNA give an advantage in tumor growth and overcome wild-type mtDNA in the promotion of tumors. We will now discuss how to increase mutant mtDNA (Figure 4).

The high frequency of mtDNA alterations in cancer and their presence in the early stages of disease could be exploited as clinical markers for early cancer detection (Fliss et al., 2000). ROS may be a cause for inducing the mutations. Superoxide anion radicals (O_2^-) are derived

from oxygen molecules by accepting electrons that leaked from the respiratory chain. In turn, they are converted into hydrogen peroxide spontaneously or by the catalytic activity of manganese-dependent superoxide dismutase (Mn-SOD). The resulting hydrogen peroxide is then converted into a hydroxyl radical by Fenton reaction in the presence of Fe^{2+} or Cu^+. Hydroxyl radicals are the most reactive and toxic, possessing the potential to oxidize lipids, proteins and DNA, leading to somatic mutations. Multiple and progressive steps of mutations in genes including oncogenes and tumor suppressors are considered to contribute to neoplatsic transformation. Thus, hydroxyl radicals are a strong candidate as an inducer of cancer. Superoxide and hydrogen peroxide are sources of hydroxyl radicals, while superoxide and hydrogen peroxide play several important roles by activating many signals for regulating homeostasis (Lee et al., 1998; Evans et al., 2000; McCord, 2000; Michiels et al., 2002). In particular, superoxide and hydrogen peroxide induce apoptosis signals (Matsuzawa et al., 2005), which may protect against cancer. Thus, one must remember that ROS may have a dual role as an inducer of as well as a protector against cancer depending upon the development stage of cancer.

Drift of mutant mitochondrial DNA toward homoplasmy
Many mutant mtDNAs found in cancer cells exhibit homoplasmy. It is impossible to mutate all mtDNA molecules at once. Then it follows, that mutant mtDNA must overcome the wild-type DNA in a cell. Fusion between cancer cells with and without mutant mtDNA resulted in homoplasmy (Polyak et al., 1998), suggesting the mutant mtDNA had overcome the wild-type in a cell. On the other hand, in the case of mutant mtDNA derived from a patient with a MELAS subgroup, heteroplasmic mutant mtDNA with a point mutation at nucleotide position 3243 was drifted toward homoplasmy in cybrids (Yoneda et al., 1992). However, the results differed among cybrids with different nuclear backgrounds (Dunbar et al., 1995). Thus, the behavior of mtDNA toward homoplasmy depends upon the nucleus.

When mtDNA with a long deletion was transferred into cybrids under the HeLa nucleus from a fibroblast, the deletion mutant gradually increased. However, when a galactose medium lacking glucose was supplied, cybrids with the deletion mutant increased by only a certain extent probably due to a low-energy production (Hayashi et al., 1991a). In this case, the deletion mutant mtDNA may be advantageous in mtDNA replication, and a sufficient energy production by glycolysis does not affect cell growth in glucose-rich medium. On the other hand, the mutant mtDNA gives a disadvantage to cell-growth in glucose-deficient medium because sufficient energy cannot be produced by glycolysis as well as oxidative phospholylation. Thus, the balance between mtDNA replication and cell proliferation seems to determine the ratio of mutant and wild-type mtDNA. The molecular mechanism of this drift is unknown. One

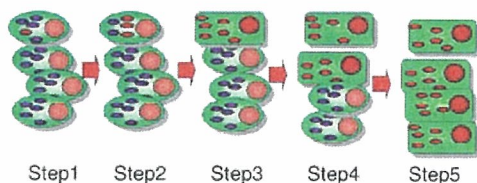Step1 Step2 Step3 Step4 Step5

Figure 4 A model explaining the increase in mutant mitochondrial DNA (mtDNA) during the development of cancer. Step 1: A mutation occurred in an mtDNA molecule, where blue and red circles indicate wild-type mtDNA and mutant mtDNA, respectively. Step 2: Mutant mtDNA increased toward homoplasmy in a cell. Step 3: Mutant mtDNA reached homoplasmy. During the increase in mtDNA, tolerance to a low-energy condition may be achieved. Step 4: Cells with homoplasmic mutant mtDNA overcome cells with wild-type mtDNA. Since cells with mutant mtDNA are insensitive to apoptosis, cells with wild-type mtDNA relatively decrease by apoptosis. Step 5: Cells containing homoplasmic mutant mtDNA predominantly remained.

possibility is a replication advantage of mutant mtDNA; alternatively, mutant mtDNA may increase by the clonal expansion of a mutant mtDNA molecule. Since the mechanism of mtDNA replication is under reconsideration (Yasukawa et al., 2005), the molecular mechanism of how mutant mtDNA increases during cancer development will be understood in the near future.

Apparent increase of the growth of mutant cybrids, but no proliferation advantage
Next, we consider how mutant cells with homoplasmic mutant mtDNA overcome wild-type cells. Mutant cells with defective mitochondria should be disadvantageous in an energy production by declining oxidative phosphorylation. However, upregulation glycolytic pathway may be complementary to the increase of mutant mtDNA in a cell (Xu et al., 2005). This apparent growth is faster in mutant cybrid cells than wild-type cells. To confirm the growth advantage of mutant cybrids, mutant and wild-type cybrids were mixed in a 1:1 ratio and the relative proportions of the mutant and wild-type mtDNAs were assessed in the mixed cultures. As a result, the relative content of mutant mtDNA progressively increased and eventually replaced the wild-type mtDNA.

To exclude the possibility that secondary mutations in nuclear genes led to a growth advantage in culture, the nuclear version of *MATP6* was introduced into mutant cybrids, and the resulting transfectants grew significantly slower than control mutant cybrids mock transfected with an empty plasmid. Conversely, when the mutant version was transfected into wild-type cybrids, the resulting transfectants increased significantly faster than control mock-transfected wild-type cybrids. Moreover, to confirm the effect of the transfection of the nuclear version of *MATP6* on the transfectant increase, we mixed the transfectants and mock transfectants in a 1:1 ratio. The number of *NuATP6* FLAG-positive cybrids decreased relative to the mock transfected. Conversely, there was an increase in the number of mutant FLAG-positive cybrids as compared to the mock transfectants in mixed cultures. 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 growth and override the effect of the mtDNA genotype.

There was no marked difference in Ki-67-positive cells (a proliferation marker) or in the cell cycle profiles between mutant and wild-type cybrids. Moreover, there was no difference in Ki-67-positive tumor cells derived from cybrids. These results indicate that the apparent growth advantage is not due to the enhancement of cell proliferation.

Lower frequency of apoptosis of mutant cybrids in vitro *and* in vivo
Mutant cybrids with mutant mtDNA or cybrid transfectants with the nuclear version of mutant *MATP6*

exhibit alterations in energy metabolism. Then the question is why cells with an energy disadvantage increased faster. Even when the glycolytic pathway is enhanced, the decline in oxidative phosphorylation should be more predominant in energy metabolism. Nevertheless, no difference in the frequency of the Ki-67-positive cells as a proliferation index among cybrid clones was seen. Additionally, the cell cycle was not accelerated by the decline of energy production. Thus, mutant mtDNA did not confer an advantage in cell proliferation. To explore the molecular mechanism underlying the growth advantage in tumors, we hypothesized that mutant mtDNA may protect cells from apoptosis, and therefore the growth advantage of mutant cybrids may be a consequence of increased survival. To test this hypothesis, apoptosis was shown by three independent methods: terminal deoxy transferase uridine triphosphate nick end labeling (TUNEL) staining, DNA fragmentation and sub-G1 population analysed by flow cytometry. The induction of apoptosis in wild-type cybrids was seen in tumors derived from wild-type cybrids than mutant cybrids.

Relationship of apoptosis with mutant mitochondrial DNA

Apoptosis by energy crisis
Some reports indicate that mutant mtDNA induces apoptosis *in vitro* and *in vivo*, whereas other reports suggest that cells possessing mutant mtDNA become tolerant to apoptosis. Thus, the relationship between apoptosis and mutant mtDNA may be confusing. However, bioenergetic crisis in these cells may be at the core of this discrepancy. When decline in energy production reaches the minimum requirement, cells cannot survive. Several mutations derived from mitochondrial disease severely affect energy production, leading to a pro-apoptotic effect. Fibroblasts isolated from patients with a subtype of mitochondrial diseases, Leigh syndrome, undergo apoptosis in glucose-deficient medium (Geromel et al., 2001), probably because neither glycolysis nor oxidative phosphorylation produces sufficient energy. The fibroblasts detached from the dish and underwent apoptosis even in glucose-rich medium. When cybrid cells carrying mutant mtDNA derived from a Leber's hereditary optic neuropathy (LHON) patient were cultured in glucose-deficient medium, they underwent apoptosis (Ghelli et al., 2003). Cells with pathogenic mutant mtDNA cannot survive without a sufficient energy supply, thus apoptosis in this case seems to be a consequence of a bioenergetic crisis.

Mice with transient mtDNA depletion in various tissues were obtained by knocking out the *Tfam* gene. Apoptosis was studied in embryos with homozygous disruption of the Tfam gene and tissue-specific Tfam knockout animals exhibited a severe respiratory chain deficiency in the heart. Massive apoptosis was found in Tfam knockout embryos on embryonic day 9.5.

Moreover, apoptosis was increased in the heart of tissue-specific Tfam knockouts (Wang et al., 2001). Additionally, mice expressing a proofreading-deficient version of mitochondrial DNA polymerase γ accumulated mtDNA mutations, and the levels of the accumulated mutations correlated with the induction of apoptotic markers (Kujoth et al., 2005; Trifunovic et al., 2005). These data provide in vivo evidence that the respiratory chain deficiency predisposes cells to apoptosis.

Development of tolerance to apoptosis by mutant mitochondrial DNA

An immortalized human fibroblast cell line carrying wild-type mtDNA underwent apoptosis in a high concentration of molecular oxygen. In contrast, its $\rho0$ exhibited a marked resistance to apoptosis (Yoneda et al., 1995). Additionally, $\rho0$ cells showed a stronger resistance to irradiation than the other two cell lines (Tang et al., 1999). $\rho0$ cells were more resistant to apoptosis-inducing agents than parental cells in vitro (Singh et al., 1999; Wang et al., 2001). In these experiments, parental cells or the other cell lines were used as controls. One should bear in mind that it is highly possible to introduce considerable mutations into nuclear genes of $\rho0$ cells by long exposure to EtBr during the isolation of a $\rho0$ cell line. Nevertheless, these consistent results obtained from various lines of research suggest that they may be negligible.

As mentioned above, in vivo $\rho0$ or mtDNA-mutant cells are more sensitive to apoptosis, while $\rho0$ cells in culture are more resistant to various apoptotic stimuli. This discrepancy may be explained as follows: cultured $\rho0$ cells may adapt to cope with the bioenergetic crisis conditions perhaps because by enhancing the activity of the glycolytic pathway. However, in vivo cells cannot adapt in response to a transient depletion. The process of malignant transformation usually takes a long time. Thus, cancer cells selected during this process are likely better adapted to cope with the energetic demands. Since cybrid cells were derived from $\rho0$ cells, they should also be fully adapted. Thus, mtDNA-mutant cybrids are more resistant to apoptosis induced by various stimuli. It remains unclear why $\rho0$ and mtDNA-mutant cybrid cells are more resistant to induction of apoptosis at the molecular level.

To understand the molecular mechanism underlying the association between decline of an energy production and sensitivity to apoptosis, it is important to know the expression profiles of nuclear genes. Transient depletion of mtDNA or treatment with mitochondrial poison CCCP initiates mitochondrial stress signaling, which operates through altered Ca^{2+} homeostasis. In myoblasts and human lung carcinoma cells, mitochondrial stress signaling activates calcineurin and a number of Ca^{2+}-responsive factors including activating transcription factor, nuclear factor of activated T cells, CCAAT enhancer-binding protein/δ and cAMP-responsive element binding protein. Additionally, protein kinase C and mitogen-activated protein kinase are also activated. Nuclear gene targets including those involved in Ca^{2+}

storage/release, glucose metabolism, oncogenesis and apoptosis are upregulated. Mitochondrial stress in both the myoblasts and lung carcinoma cells induced morphological changes and invasive phenotypes (Biswas et al., 2005). It is important to investigate the molecular mechanism underlying tolerance to apoptosis from the aspects of nuclear gene expression and regulation.

Mitochondrial DNA mutations confer tolerance to anticancer drugs

Emergence of drug resistance is a critical problem for chemotherapy, but the mechanisms underlying this phenomenon are not completely understood. Most anticancer drugs are inducers of apoptosis. As mentioned above, mutant mtDNA in cybrids confer resistance to apoptosis. When cybrids were treated with cisplatin, a typical anticancer drug, mutant cybrids were more resistant to apoptosis than the wild-type (Shidara et al., 2005).

Another report supports our results that mutant cybrids are more resistant to anticancer drugs. $\rho0$ cells are extremely resistant to adriamycin and photodynamic therapy (Singh et al., 1999) and cisplatin (Qian et al., 2005). Tolerance to etoposide was found even by the transient depletion of mtDNA (Biswas et al., 2005).

Interestingly, ATP synthase is downregulated in some 5-fluorouridine (5-FU)-resistant cells, which also showed decreased ATP synthase activity and reduced
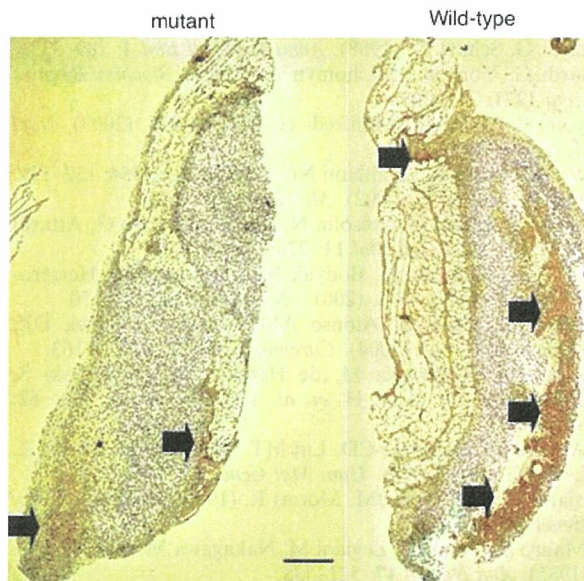

Figure 5 Tolerance to cisplatin in tumors derived from cybrids possessing mutant mitochondrial DNA (mtDNA). Cybrids carrying mtDNA with a homoplasmic mutation in the cytochrome c oxidase subunit I gene were transplanted into nude mice. Cisplatin was administrated to the nude mice and the tumors were evaluated for apoptosis by the terminal deoxy transferase uridine triphosphate nick end labeling (TUNEL) method. Arrows indicate massive TUNEL-positive areas. Right panel indicates a tumor derived from wild-type cybrids, while the left panel from mutant cybrids. Scale bar: 0.5 mm.

intracellular ATP content (Shin *et al.*, 2005). When 5-FU sensitivity was compared with ATP synthase activity in six different human colon cancer cell lines, positive correlation was observed. The bioenergetic dysfunction of mitochondria has been reported as a hallmark of many types of cancers; that is, downregulation of ATP synthase β-subunit expression in liver, kidney, colon, squamous esophageal, and lung carcinomas, as well as in breast and gastric adenocarcinomas (Cuezva *et al.*, 2002, 2004; Isidoro *et al.*, 2004). Thus, decreased ATP synthase activity may be associated with resistance to some anticancer drugs. Tolerance to anticancer drugs is due not only to the decline of ATP synthase activity, but rather to alterations in mitochondrial activity. We constructed cybrids harboring mutant mtDNA derived from pancreatic cancer cells. Mutant mtDNA have a mutation at cytochrome *c* oxidase or the subunit 4 of complex I (ND4). The cybrids were strongly resistant to cisplatin and 5-FU *in vivo* and *in vitro* (Figure 5) (manuscript in preparation).

Mitochondria are directly involved in apoptosis by releasing factors involved in the initiation, regulation and execution of apotosis (Ohta, 2003). Members of the bcl-2 family regulate apoptosis on mitochondria. To understand the link between mitochondrial dysfunction and apoptosis at the molecular level, the response profiles of these factors to mitochondrial dysfunction will be investigated. Alternatively, it seems important to highlight cross-talk between the nucleus and mitochondria. The expression of apoptosis regulators may be influenced by dysfunctional mitochondrial through a decrease in ATP, a Ca^{2+} signaling or an increase in lactate or pyruvate by enhancing glycolysis.

Concluding remarks

Methods that had been developed to clarify the responsibility of mutant mtDNA in mitochondrial diseases were applied to investigate its role in tumorigenesis. Cybrids containing a common nucleus and mtDNA with a pathogenic mutation revealed an apparent advantage in growth when transplantated into nude mice. This advantage is related to alterations in mitochondrial activity, but not secondary mutations in the nuclear genome. However, tumors formed by cybrid transplantation may differ in many ways from naturally occurring cancers. More careful investigation will be required to understand the veritable roles of mtDNA in cancer. Additionally, we addressed the role of mutant mtDNA in conferring tolerance to anticancer drugs. By understanding the molecular mechanism of how to gain tolerance, mtDNA will be extensively investigated for clinical application in the light of apoptosis.

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Laboratory Investigation

Prognostic significance of the immunohistochemical index of survivin in glioma: a comparative study with the MIB-1 index

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Summary

Objective: Survivin has been identified as a protein expressed in cancer cells and a member of the inhibitor-of-apoptosis protein family. Recent studies suggest that the expression of survivin increases during the G2/M phase of the cell cycle, and may be used in clinical prognosis. We examined whether survivin expression in human gliomas would be a correlative of prognosis.

Methods: We prepared polyclonal anti-survivin serum to establish a survivin index for stained sections, using an immunohistochemical procedure, according to the method used for scoring MIB-1 index, and then stained 29 paraffin-embedded sections from surgical specimens of 29 patients who were classified into three grades of World Health Organization with the mean age of low grade astocytoma (grade II) being 34.7; anaplastic astrocytoma (grade III), 48.8; and glioblastoma multiform (grade IV), 58.4.

Results: On staining with the anti-survivin antiserum, all specimens contained positive cells, but the survivin index was heterogeneous among grades. The mean percentage of immunoreactive cells in each specimen was 70.0% (SD 18.2%) in grade II, 81.3% (16.5%) in grade III, and 85.0% (13.6%) in grade IV. Then we compared the survivin index to the MIB-1 index and found that in low-grade gliomas (grade II and III), the difference in survival times between the high and low survivin indexes was significant ($P = 0.007$), whereas that between the high and low MIB-1 indexes was not significant ($P = 0.092$).

Conclusion: Survivin is more sensitive marker than MIB-1 for the evaluation of low-grade gliomas in that it helps to predict patient survival. Much larger glioma patient series are needed to validate the findings of our limited study.

Introduction

Glioma, the most common neoplasm in the human brain, includes tumors derived from astrocytes, oligo-dendrocytes, ependyma, and choroid plexus epithelium. Gliomas are histologically divided into four grades according to World Health Organization (WHO) guidelines. High grade glioma, GBM (glioblastoma multiform; grade IV), has the worst prognosis with a median survival time of about 12 months, even after surgical resection, radiation therapy, and chemotherapy. Of lower grade gliomas, patients with anaplastic astro-cytoma (grade III) have an average survival of 3 years, whereas patients with low grade astrocytoma (grade II) have a better prognosis, with at least 5 years median survival [1]. However, the outcome for patients with anaplastic and low grade astrocytoma is highly variable [2,3]. A significant number of patients rapidly develop malignant glioma [4,5]. It has been reported that the proportion of tumor cells with abnormal p53 immuno-reactivity increases in astrocytomas as they undergo malignant progression [6,7]. Thus, an accurate diagnosis is important from both clinical and experimental per-spectives.

To date a number of methods for predicting the prognostic subgroups of glioma patients have been de-scribed [2,3]. One of the methods, the immunohisto-chemical determination of proliferative activity with the monoclonal antibody MIB-1 against Ki-67, which is a nuclear antigen, has been widely demonstrated to be clinically useful in distinguishing the biologic behavior of many tumors [5,8]. However, conflicting results from the utilization of Ki-67 as a prognostic marker for gli-oma especially for low grade glioma, have been reported [9–11].

Survivin is a member of the inhibitor-of-apoptosis protein (IAP) family and has been implicated in both the inhibition of apoptosis and regulation of mitosis [12,13]. IAPs are characterized by the presence of one to three baculovirus inhibitor apoptotic protein repeat domains, which often function as an inhibitor of the cell death process. The survivin gene is of the telomeric position on chromosome 17, to band q25 and its expression is determined in the developing embryo and in rapidly dividing cells including many human cancers [14,15]. Previous studies demonstrated that survivin is expressed at G2/M in a cell-cycle dependent manner and associ-ated with kinetochores of metaphase chromosomes and

the central spindle midzone at anaphase, suggesting that survivin participates as a chromosomal passenger protein in cleavage furrow formation [13,16]. *In vitro*, it bound polymerized microtubles and a putative tubulin-binding domain was identified by mutational analysis. Forced expression of survivin counteracted cell death induced by various apoptotic stimuli, whereas interference with the expression or function of survivin by a dominant negative form or antisense of survivin caused spontaneous apoptosis and multiple cell division defects, suggesting that survivin acts both as a mitotic regulator and as a cytoprotective factor at cell division [16,17].

Overexpression of survivin has been observed in most human cancers including glioma by analysis of its mRNA and/or protein, whereas a low level of survivin expression is detected in normal tissues [18,19]. Recently, Chakravarti et al. has reported that the quantitative determination of survivin by Western blot analysis is useful for prognosis in human glioma [20]. However, in their study, survivin was not detected in 36% of gliomas. Thus, the aim of this study was to evaluate the histological determination of survivin with a sensitive anti-survivin anti-serum in a consecutive series of low- and high-grade gliomas to assess the prognostic significance of survivin expression. We here demonstrated that all gliomas expressed survivin and that the percentage of immunoreactive cells (survivin index) had a strong reverse association with survival time of patients. In particular, the survivin index correlated with patient survival in low grade gliomas.

Materials and methods

Cell culture

Human fibroblast, SK-N-SH (human neuroblastoma), HeLa (human uterin cervix epitheloid carcinoma), SNB-19 (human glioblastoma), FDCP-1 (mouse bonemarrow), and CHO K-1 (Chinese hamster ovary) were cultured in Dulbecco's modified Eagle medium/Ham's F12 (Life Technologies, Rockville, MD, USA) containing 10% fetal bovine serum with penicillin-streptomycin. Jurkat (human T cell leukemia) cells were cultured in RPMI1640 (Life Technologies) containing 10% fetal bovine serum with penicillin-streptomycin. Free from microbiological contamination was confirmed with PCR procedure (Takara, Tokyo, Japan).

Antiserum preparation

Survivin cDNA was obtained from SK-N-SH by the reverse transcription-PCR method with a pair of primers, 5'-NNGAATTCAATCCATGGCA GCCAGCTG and 5'-NNGAATTCAATGGGTG CCCCGACGTTG (N means any of the four nucleotides). On digestion with *Eco*RI, the resultant fragment was inserted into pProEx3 GST (Life Technologies) and sequenced to confirm accurate the full-length survivin cDNA. Overexpression and purification of a fusion protein of survivin with glutathione S-transferase (GST) in *Escherichia coli* were performed by a method described previously [21]. Rabbits were immunized with the purified protein with Freund's adjuvant of a mixture of 1:1 (Life Technologies).

Western blot analysis

Total cell lysates were separated on a 12.5% SDS-polyacrylamide gel electrophoresis and transferred onto poly-vinyl difluoride membrane (NEN, Boston, MA, USA). The membrane was blocked with 10% fetal bovine serum in Tris buffered saline (TBS) for 1 h, and then incubated with diluted anti-survivin antiserum in TBS with 0.2% Tween-20 (TBS-T) at 4 °C overnight. After a wash with TBS-T, the membrane was incubated with alkaline phosphatase-conjugated anti-rabbit IgG (Cappel, Aurora, OH, USA) and specific bands were visualized with AttoPhos substrate (Roche, Mannheim, Germany).

Immunocytochemistry

HeLa and human fibroblast cells were cultured on 4-well plastic dishes (SonicSeal slide, Nalge Nunc, Rochester, NY, USA). The cells were rinsed with phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde in PBS. After a wash with PBS, the cells were incubated 30 min in 0.2% Triton X-100, 30 min in a blocking buffer (3% bovine serum albumin and 3% goat serum in PBS), and overnight at 4 °C in a blocking buffer containing anti-survivin antiserum (1:250). After another wash with PBS, the cells were incubated in a blocking buffer containing BODIPY FL goat anti-mouse IgG (1:500, Molecular Probes, Eugene, OR, USA) for 1 h and imaged with a confocal scanning microscope, the FLUOROVIEW FV/300 (Olympus, Tokyo, Japan).

Tissue processing and clinical data

There were 37 gliomas from 1994 to 2001 and 29 of these were suitable for this analysis. We prepared glioma samples from 29 patients [17 males and 12 females, age from 6 to 80 (mean 45.4) at the time of diagnosis; Table 1] who were operated with craniotomy, excepted biopsy, with informed consent. The samples were selectively collected from center zone of the tumor, quickly frozen, kept at −80 °C until used, fixed with formalin, and embedded in paraffin. Sections (4 μm) were used for immunohistochemistry and the classification of grades according to WHO guidelines diagnosed by two or more pathologists and neurosurgeons. Clinical data were obtained from the hospital records including age, sex, and survival time from the initial operation. Follow-up was available for all patients.

Immunohistochemistry

For immunostaining with anti-survivin antiserum, the 4-μm sections were stained using the Ventana NexES

Table 1. Characteristics in glioma patients[a]

No.	Age	Sex	WHO grade	MIB-1 index (%)	Survivin index (%)	Resection	Location	Depth	Survival days[b]	Out-come
1	30	male	2	5	85	subtotal	Temporal	sup[d]	2618	alive
2	40	female	2	5	75	total	Frontal	sup	2280	alive
3	10	male	2	n.d.[c]	65	total	Cerebellum	sup	2268	alive
4	26	female	2	5	65	total	Frontal	deep	2025	alive
5	27	female	2	25	80	subtotal	Pons	deep	246	dead
6	45	male	2	0	45	total	Frontal	deep	1550	alive
7	55	male	2	10	95	total	Frontal	deep	777	dead
8	29	male	2	n.d.	40	total	Pariental	sup	212	dead
9	50	male	2	5	80	subtotal	Frontal	deep	62	dead
10	46	male	3	5	65	subtotal	Frontal	deep	1505	alive
11	12	female	3	15	90	total	Frontal	deep	825	dead
12	6	female	3	35	90	subtotal	Pons	deep	150	dead
13	64	male	3	n.d.	85	subtotal	Frontal	sup	636	dead
14	64	male	3	0	85	subtotal	Frontal	deep	438	dead
15	21	male	3	5	35	total	Pons	deep	1173	dead
16	58	male	3	n.d.	95	subtotal	Frontal	deep	220	dead
17	23	male	3	15	85	total	Frontal	deep	425	alive
18	69	male	3	20	95	subtotal	Temporal	deep	336	dead
19	74	female	3	5	80	subtotal	Pariental	sup	102	alive
20	54	female	3	5	85	subtotal	Pariental	deep	180	dead
21	46	male	3	5	85	total	Frontal	deep	415	dead
22	54	male	4	n.d.	55	subtotal	Frontal	deep	374	dead
23	31	male	4	n.d.	80	subtotal	Frontal	deep	118	dead
24	78	male	4	n.d.	95	subtotal	Frontal	sup	47	dead
25	54	female	4	20	100	total	Occipital	sup	247	dead
26	80	female	4	55	90	subtotal	Frontal	deep	48	dead
27	79	female	4	55	90	subtotal	Frontal	deep	422	dead
28	11	female	4	n.d.	85	total	Cerebellum	sup	115	dead
29	80	female	4	55	85	subtotal	Frontal	sup	127	dead

[a]All samples were resected with craniotomy, excepted biopsy samples. [b]Survivial days are from the first operation. [c]Not determined. [d]Superficial.

Staining System (Ventana, Tucson, AZ, USA) and all products without the anti-survivin antiserum needed for subsequent steps were supplied by the manufacture (Ventana). Sections were deparaffinized and heated with CC1 solution (denature buffer; Ventana) for 1 h. After 32-min incubation at 37 °C with the anti-survivin antiserum (1:250), sections were further incubated for another 10 min at 37 °C with a secondary biotinylated antibody and then with avidin-peroxidase for another 10 min; 3′, 3-diaminobenzidine was used as the chromogen. Slides were counterstained in Mayer hematoxylin, dehydrated, and mounted. Immunostaining with MIB-1 (1:50 dilution, Immunotech, Westbrook, ME, USA) for detection of Ki-67 antigen was performed as described previously [4,22]. Stained sections were observed under the microscope. Survivin and MIB-1 indexes were determined as the percentage of immunostained cells per 200 cells × 5 fields per section.

Statistical analysis

Statistical analyses were performed using Stat View for Macintosh Version 5.0 (SAS Institute Inc., Cary, NC, USA). The survivin index, the MIB-1 index, and survival times were subjected to linear regression analysis. Survival was plotted, and survival time was estimated by the Kaplan-Meier method. The survival times and the strength of associations between categories were compared with the log-rank test (Mantel-Cox) for univariate analysis. For multivariate analysis, tumor location (temporal, frontal, pariental, occipital, pons or cerebellum), depth (deep or superficial), age (young $\leq$ 20 year-old, 20 < middle < 65, or 65 $\leq$ old), survivin index (higher 17 samples, or lower 12 samples), and MIB-1 index (higher 10 samples or lower 11 samples) were compared with survival times. A P < 0.05 was considered significant.

Results

Antisera specific to the survivin protein

To examine the expression of survivin in patients immunohistochemically, rabbit antisera were raised against GST-survivin fusion protein. The diluted sera (1:10000) specifically stained only major 16.5-kDa and minor 14-kDa bands in Western blots of 3 µg total cell lysate from cultured GBM, SNB-19 (Figure 1a, Lane 1–2). A 16.5-kDa specific band of survivin was also detected in Western blots of 1 µg lysate from other cultured cells (Figure 1a, Lane 3–5). Immunocytochemical staining of HeLa cells with the obtained antiserum revealed that survivin immunoreactivity was associated with the centrosome, mitotic spindle and midbody (Figure 1b), as previously described [13,16,17].
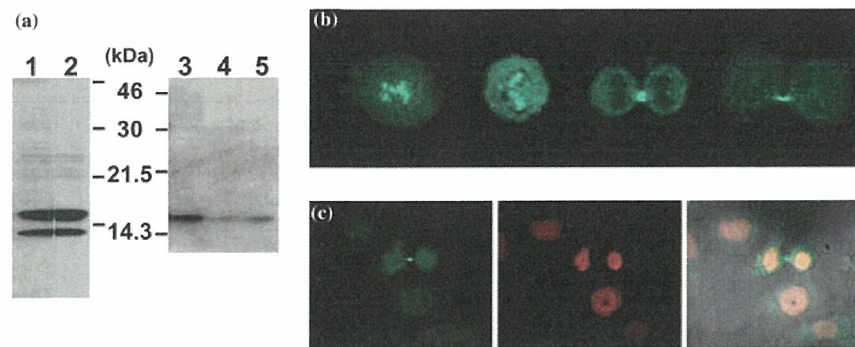
Figure 1. Characterization of anti-survivin antiserum. (a) Western blotting analysis for survivin in each cell lysate from SNB-19 (glioma, Lane 1 and 2), FDCP-1 (mouse bone marrow, Lane 3), Jurkat (Lane 4), and CHO K-1 (Chinese hamster ovary, Lane 5). Three µg per lane (Lane 1 and 2) or 1 µg per lane (Lane 3–5) of lysate was subjected and stained with 1:5000 diluted sera (Lane 1, 3–5) or with 1:10000 diluted sera (Lane 2). (b) HeLa cells in mitosis were immunohistochemically stained with 1:1000 diluted sera. Original magnification, × 600. (c) Human primary fibroblasts were immunohistochemically stained with 1:1000 diluted sera. Left panel; stained with anti-survivin serum, middle; with propidium iodine, and right panel; merge. Original magnification, ×600.

Furthermore, survivin localized in the midbody of primary cultured fibroblast was also detected as shown in Figure 1c, suggesting that the antiserum preparation immunodetects survivin in not only malignant tumors but also normal replicating cells, and is strong enough in immuno-reactivity and purity for further experiments.

Distribution patterns of survivin in glioma

Automated immunohistochemistry was performed on paraffin-embedded sections prepared from surgical specimens with anti-survivin antiserum (1:250) using the Ventana IHC staining system. Stained sections from glioma patients in various histological grades are shown in Figure 2A, C, and E. No positive immunoreactivity was detected without antiserum, and survivin expression was undetectable around gliosis (Figure 2G) and in normal tissues (Figure 2H). All specimens (n = 29) from glioma patients contained positive cells, but the percentage of positive cells (designated as the survivin index) was heterogeneous among grades, which were determined with histopathological diagnosis (Figure 3a). The mean percentage of immunoreactive cells in each specimen was 70.0% in low grade astrocytoma (grade II, 9 specimens), 81.3% in anaplastic astrocytoma (grade III, 12 specimens), and 85.0% in GBM (grade IV, 8 specimens). Thus, the immunoreactivity of survivin is a highly sensitive marker for glioma.

Progonostic value of survivin index for glioma

To evaluate the prognostic value of the survivin index, the correlation of survival time of patients with the survivin index for all grades of glioma was investigated using a scatter plot diagram (Figure 4a). An increasing percentage of survivin-positive cells was reversely associated with the survival time ($P = 0.049$). The patients were further divided in two groups, high (index ≥ 80) and low (80 > index), and then the survival curve of the groups was calculated by the Kaplan-Meier method (Figure 5a). Survival times were significantly shortened ($P = 0.003$) for patients whose survivin index was high

(mean survival time = 349 days) compared with those whose index was low (mean survival time = 953 days).

Lower correlation between survivin and MIB-1 indexes in low-grade gliomas

Ki-67 antigen, which is detected by MIB-1 antibody, is a nuclear protein complex and the percentage of MIB-1- positive cells (MIB-1 index) is widely used for the diagnosis of many malignant tumors including glioma [5,8,23]. Then, we investigated the correlation between survivin and MIB-1 indexes in consecutive sections (Figure 2). A linear positive correlation between them was not significant, but weak correlation was noted essentially (Figure 6, $P = 0.065$), whereas an increasing percentage of MIB-1-positive cells was still associated with worse survival (Figure 4b, $P = 0.037$). However, as shown in Figure 3b, MIB-1 indexes in anaplastic astrocytoma were shown to be as low as those in low grade astrocytoma, whereas survivin indexes in anaplastic astrocytoma were similar to those in GBM, indicating that the survivin index would be a more sensitive marker for low-grade gliomas than the MIB-1 index. Then, patients were divided into two groups based on the MIB-1 index, high (index ≥ 10) and low (10 > index), and then the survival curve of each group was obtained by the Kaplan-Meier method. In total, survival times were significantly shortened (Figure 5b, $P = 0.033$) for patients whose MIB-1 index was high (mean survival time = 398 days) compared with those whose index was low (mean survival time = 821 days). However, in low-grade gliomas, the difference in survival times between the high and low MIB-1 indexes was not significant (Figure 5d, $P = 0.092$). At that time, in low-grade gliomas, the difference in survival times between the high and low survivin indexes was still significant (Figure 5c, $P = 0.007$).

Finally, we compared the difference in survival times among age of patients, tumor location, depth, the survivin index, and the MIB-1 index by a multivariate analysis. The suvivin index was significantly associated
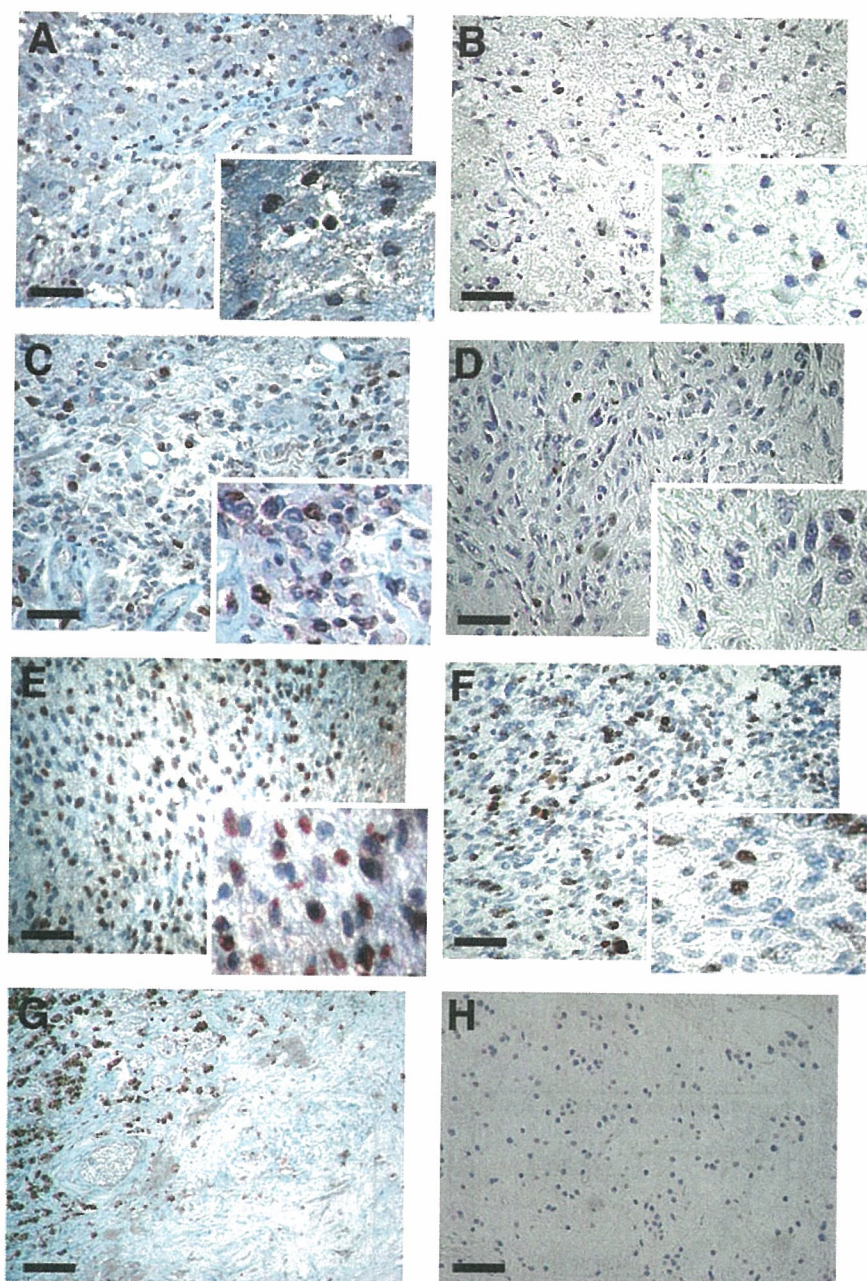
Figure 2. Immunostaining of gliomas. Immunohistochemical staining for surgical sections staining with 1:250 diluted anti-survivin serum (A, C, E, G and H), using the Ventana NexES Staining System (Ventana, Tucson, AZ). Immunohistochemical staining for surgical sections with 1:50 diluted anti-Ki-67 serum (B, D and F). A and B; low grade astrocytoma. C and D; anaplastic astrocytoma. E, F and G; GBM. G; local invasion of GBM and around gliosis. H; normal brain tissue. Original magnification, ×200 and ×400 (insets). Bars are 100 μm.

with the survival times ($P = 0.036$), while other characteristics were not (location: $P = 0.922$, depth: $P = 0.402$, age: $P = 0.866$ and MIB-1 index: $P = 0.404$). Taken together, these findings strongly suggest that the survivin index is a useful tool for the prognosis of histologically low-grade gliomas.

Discussion

In this study, we demonstrated that gliomas highly expressed survivin and that the percentage of immunoreactive cells (survivin index) had a strong inverse association with the survival of patients. In particular, the survivin index correlated with the survival of low grade gliomas, suggesting that the index would be useful for a clinical prognosis.

Our antiserum raised against the GST-survivin fusion protein was highly specific and sensitive to the survivin protein (Figure 1). Western blotting of cell lysates revealed that both the 16.5-kDa full- length and 14-kDa alternatively spliced products were expressed in cultured GBM SNB-19 (Figure 1a, Lane 1–2). We used a survivin-specific RT-PCR and identified that a 14-kDa band
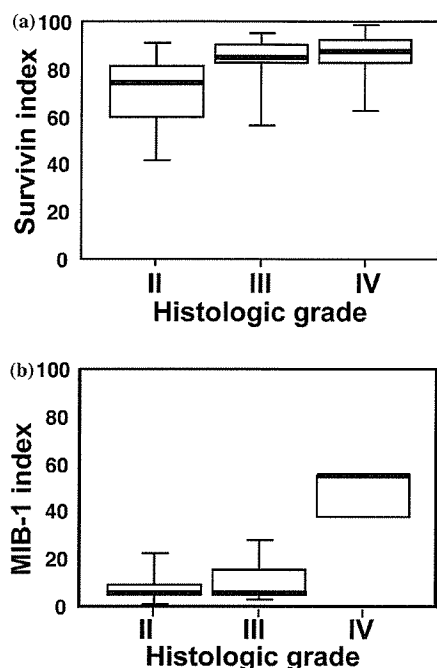
Figure 3. Correlation of histological grade with indexes. Correlations of survivin (a) and MIB-1 (b) indexes vs. histological grade are demonstrated. The difference between grade II and grade III is more obvious in the survivin index than MIB-1 index. Data are presented as the median (solid line), 25th and 75th percentiles (vertical boxes) and 10th and 90th percentiles (error bars).
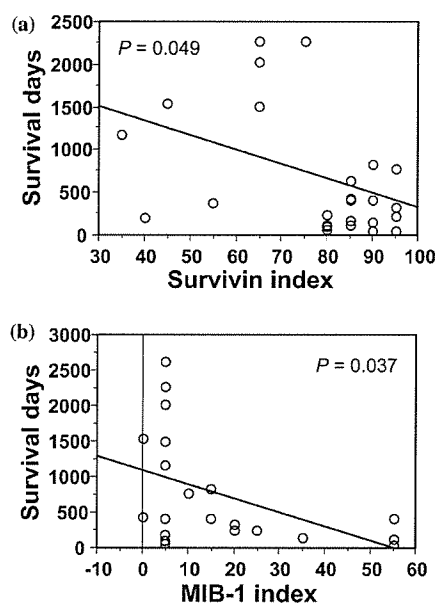

Figure 4. Correlation of survivin and MIB-1 indexes with survival times of patients. Survival time is the period until patients died from the first operation, the index is the percentage of immunoreactive cells in tumor cells in each surgical specimen. Survivin (a) and MIB-1 indexes (b) vs. survival time in each patient (each dot) are demonstrated.

was survivin-ΔEx3 and 10–40 % of survivin mRNA was survivin-ΔEx3 (unpublished results). However, functions of the regulatory balance between them are un-

known [21,24]. Immunocytochemistry with anti-survivin antiserum clearly demonstrated that both the immortal cell line and primary cultured fibroblasts expressed survivin in a cell-cycle dependent fashion (Figure 1b and c). However, immunological signals in highly proliferative cells, such as HeLa cells, were stronger than those in
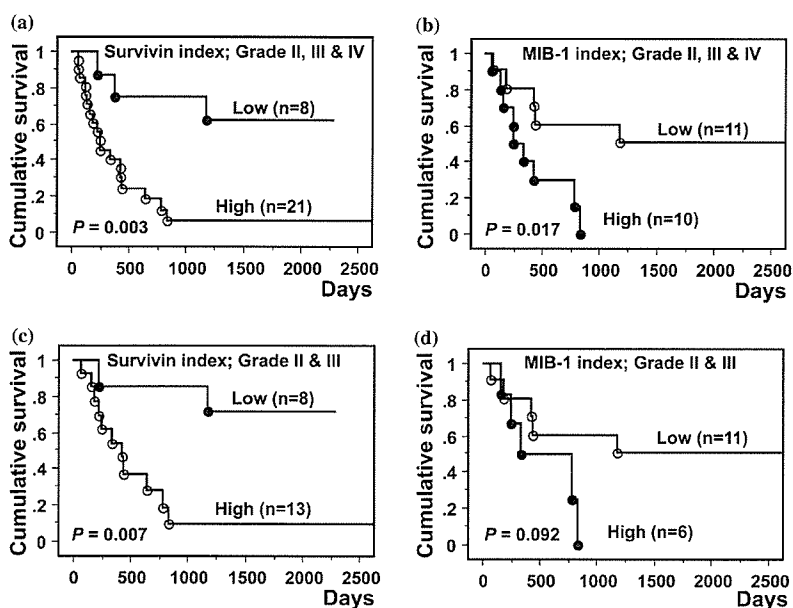

Figure 5. Kaplan-Meier plots in survivin and MIB-1 indexes. (a and c) Comulative survival rate for survivin index. Patients were separated into two groups, high (index ≥ 80) and low (80 > index). a; patients with all grade gliomas. c; all patients except those with GBM (grade IV). (b and d) Comulative survival rate for the MIB-1 index. Patients were separated into two groups, high (index ≥ 10) and low (10 > index). b; patients with all grade glioma. d; all patients except those with GBM (grade IV). Note that the survivin index is significant for survival times in glioma patients except those with GBM, but the MIB-1 index is not.
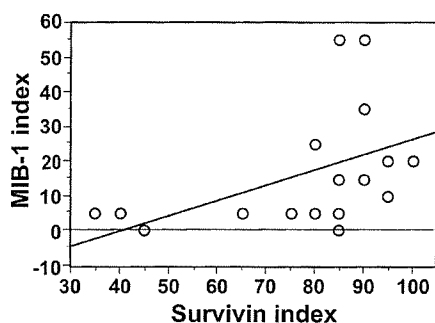
Figure 6. Correlation between the survivin index and MIB-1 index.

analyzed separately from GBM, the increased survivin index remained an important prognostic variable (Figure 5c). These results suggest that the survivin index is more significant than the histological grading to the prognosis for gliomas. Furthermore, the MIB-1 index has been reported to be clinically useful in the prognosis. However, the MIB-1 index did not predict prognostic reliability in gliomas separated from GBM (Figure 5d). Indeed, in several studies, the MIB-1 index did not correlate with survival in the low grade gliomas [9,10,23]. Finally, the survival index was confirmed to be significantly associated with the survival times by the multivariate analysis. Thus, this study proposed a possibility that the survivin index, which is correlated with the survival of low grade gliomas, will be a powerful tool for a clinical prognosis. It will be required to analyze more samples to reveal its usefulness.

primary cells. Then, the anti-survivin antiserum was confirmed for sensitivity and purity in immunohistochemical studies.

Large numbers of cells in gliomas of all grades were survivin- positive by the immunohistochemical staining (Figure 2). Positive cells having weak survivin immunoreactivity in cytosol, but not in the nucleus, were observed. Recent findings of Fortugno et al. have indicated that endogenous survivin exists in strikingly different subcellular pools, comprising a predominant cytosolic fraction and a smaller nuclear pool [13]. Therefore, an immunohistochemically detectable amount of survivin was accumulated not only in the nucleus, but also in cytosol of gliomas. In addition, the immunoreactivity of survivin is a highly sensitive marker for glioma, providing the narrow window of the survivin index compared with the MIB-1. We found that the proportion of gliomas with a high survivin index was greatest in GBM (85.0% (SD 13.6%)) and lowest in low-grade astrocytomas (70.0% (18.2%)). The survivin index in anaplastic astrocytoma (81.3% (16.5%)) was not significantly different from that in GBM. On the other hand, the MIB-1 index in anaplastic astrocytoma (11.0%) was not significantly different from that in low-grade astrocytomas (7.9%), indicating that the survivin index is a more sensitive marker than the MIB-1 index for all grades of gliomas.

It was reported that the survivin expression in neuroblastoma cell lines was strongly down-regulated during the induction of apoptosis and the expression of exogenous survivin prevented cell death. Survivin is thought to inhibit apoptosis by directly inhibiting terminal effectors caspase-3 and -7, and to maintain the normal function of the mitotic apparatus [12,15]. Thus, in glioma, survivin may enhance cell survival and proliferation, and then, its expression is associated reversely with prognostic factors.

Significant numbers of patients with anaplastic astrocytoma and low grade astrocytoma rapidly developed more malignant tumors. Since standard histological techniques do not precisely predict which tumors will undergo rapid malignant progression, it is difficult to give accurate prognostic information to patients. As shown in Figure 4a, the survivin index was associated with a significant decrease in the survival of patients nevertheless the high SD values. When gliomas were

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MIDAS/GPP34, a nuclear gene product, regulates total mitochondrial mass in response to mitochondrial dysfunction

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Summary

To investigate the regulatory system in mitochondrial biogenesis involving crosstalk between the mitochondria and nucleus, we found a factor named MIDAS (mitochondrial DNA absence sensitive factor) whose expression was enhanced by the absence of mitochondrial DNA (mtDNA). In patients with mitochondrial diseases, MIDAS expression was increased only in dysfunctional muscle fibers. A majority of MIDAS localized to mitochondria with a small fraction in the Golgi apparatus in HeLa cells. To investigate the function of MIDAS, we stably transfected HeLa cells with an expression vector carrying *MIDAS* cDNA or siRNA. Cells expressing the MIDAS protein and the siRNA constitutively showed an increase and decrease in the total mass of mitochondria, respectively, accompanying the regulation of a mitochondria-specific phospholipid, cardiolipin. In contrast, amounts of the mitochondrial DNA, RNA and proteins did not depend upon MIDAS. Thus, MIDAS is involved in the regulation of mitochondrial lipids, leading to increases of total mitochondrial mass in response to mitochondrial dysfunction.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/118/22/5357/DC1

Key words: Mitochondria, Mitochondrial mass, Cardiolipin, Mitochondrial DNA, Mitochondrial disease, Golgi apparatus

Introduction

The mitochondrion is the center of energy metabolism in eukaryotes and has recently been recognized as a multifunctional organelle (Ohta, 2003). It is involved in the regulation of apoptosis as a reservoir of signals, regulators and executioners (Kroemer and Reed, 2000; Green and Kroemer, 2004). In addition, it functions as a source of reactive oxygen species, which are believed to cause many lifestyle-related diseases, neurodegenerative diseases, cancer and aging (Kowaltowski and Vercesi, 1999; Cortopassi and Wong, 1999; Melov, 2000). Thus, mitochondria are essential in many aspects of medicine as well as cell biology.

Depending on cell type, energy demands and physiological conditions, mitochondria vary in number, mass and morphology (Attardi and Schatz, 1988; Yaffe, 1999; Collins et al., 2002; Nisoli et al., 2003). The proliferation of cells usually accompanies an increase in mitochondria. However, an increase in number of mitochondria is not distinctly coordinated with the cell cycle. For example, muscle mitochondria increase in response to exercise, independently of cell division (Brunk, 1981; Moyes et al., 1997). Exposure to a low-temperature environment or cultivation in glucose-deprived medium induces a marked increase in mitochondrial mass (Klaus et al., 1991; Weber et al., 2002). In addition, mitochondria increase in response to external stimuli with a wide range of substances including benzodiazepine, phorbol esters, calcium fluxes (Bereiter-Hahn and Voth, 1994; Vorobjev and Zorov, 1983; Muller-Hocker et al., 1986; Kawahara et al., 1991), thyroid hormones (Goglia et al., 1999) and nitric oxide (NO) (Nisoli et al., 2004). Mitochondrial numbers also increase in response to internal stimuli, such as the mitochondrial dysfunction caused by pathogenic mtDNA mutations (Schon, 2000; Wallace, 1999; Moraes et al., 1992). An increase in mitochondrial mass was observed in mitochondrial transcription factor A (*Tfam*) knockout mice, which have depleted mtDNA (Hansson et al., 2004).

As nuclear genes encode most mitochondrial proteins, including the enzymes and cofactors required for the transcription and replication of mtDNA, mitochondrial biogenesis depends on a distinct crosstalk between two physically separated genetic systems (Garesse and Vallejo, 2001). Recently, the pathway that links external physiological stimuli to the regulation of mitochondrial biogenesis and function has been studied. Several transcription/replication factors directly regulate mitochondrial genes and the

coordination of these factors into a programmed response to the environment was reported (Scarpulla, 2002).

However, the nature of mitochondrial biogenesis in response to internal stimuli is poorly understood. Mitochondrial stress results in enhanced expression of sarcoplasmic reticular ryanodine receptor-1 and some Ca^{2+}-responsive transcription factors (Biswas et al., 1999). Several tumor-specific markers are overexpressed in cells subjected to mitochondrial genetic as well as metabolic stress (Amuthan et al., 2001). Moreover, we have reported that expression of the apoptosis-mediator Fas is enhanced by dysfunctional mitochondria (Asoh et al., 1996). However, no one has reported on the mammalian factors, in response to a signal from mitochondria to the nucleus, which are involved in the stimulation of mitochondrial growth. Notably, the molecular mechanism regulating the biogenesis of mitochondrial lipids is poorly understood.

In this study, we identified factors whose expression was enhanced by depletion of mtDNA. One of them was found to increase total mitochondrial mass without a pathogenic swelling, when overexpressed. Thus, the factor is involved in the accumulation of mitochondria in response to mitochondrial dysfunction.

Materials and Methods

Cells and culture

EB8 and Ft2-11 were described previously (Hayashi et al., 1991; Hayashi et al., 1994). EB8 is a clone, derived from HeLa cells, completely lacking mtDNA, whereas Ft2-11 was constructed by transferring wild-type mtDNA into EB8 so that Ft-2-11 has the same nucleus as EB8. Stable transfectants expressing MIDAS constitutively were constructed from HeLa cells by transfection with MIDAS cDNA under the control of the CMV promoter or its empty vector (pCMV-SPORT; Life Technologies).

Stable transfectants expressing siRNA of MIDAS were constructed from HeLa cells by transfection with the pSilencer vector (Ambion) with inserts targeting MIDAS (5′-AAGCTTTTCCAAAAAAGTGG-AATGTCTGAAGGCCATCTCTTGAATGGCCTTCAGACATTCC-ACGGGATCC-3′) or a random sequence.

HeLa cells and stable transfectants were cultured in DMEM/F-12 (1:1) (Gibco-BRL) supplemented with 10% FBS and 1% penicillin/streptomycin (Gibco-BRL).

Construction of Myc-tagged MIDAS

To insert the Myc tag at the N-terminus of MIDAS, an EcoRI site was generated at the 5′ end of the MIDAS coding sequence by PCR and was cloned into the pCMV-SPORT vector. An oligonucleotide encoding MEQKLISEEDLNS (Myc tag sequence underlined) was inserted at the newly generated EcoRI site of MIDAS. To construct the Myc tag at the C-terminus of MIDAS, a BamHI site was generated at the 3′ end of the coding sequence and an oligonucleotide encoding DPEQKLISEEDL was inserted.

Differential display

Poly(A)+ RNA was purified from Ft2-11 and EB8 and reverse transcribed. Resultant cDNAs were amplified using arbitrary primer sets, followed by 5% PAGE. The gel was stained with Vistra Green (Amersham Biosciences) and visualized with a Fluoro Imager (Molecular Dynamics) (Liang and Pardee, 1992).

Antibodies

Anti-MIDAS polyclonal rabbit antiserum was raised against His-tagged MIDAS expressed in *Escherichia coli*. Anti-MIDAS antibody was affinity purified by binding to the MIDAS protein isolated by SDS-PAGE, followed by transfer onto a PVDF membrane. Anti-Tom20 and anti-Tom40 were gifts from K. Mihara, Kyushu University, Japan. Other antibodies were purchased as follows: anti-actin (clone AC-40) and anti-β-tubulin from Sigma; anti-p230 antibody and anti-Syntaxin6 from BD Biosciences; anti-Hsc70 antibody from Santa Cruz; anti-Hsp60 from MBL; anti-cytochrome c antibody and anti-Cox4 from Clontech; and anti-SDH70, anti-SDH30, anti-COX I and anti-COX II antibodies from Molecular Probes.

Immunohistochemical staining of muscle sections

Biopsy samples were obtained from the biceps brachii muscle with informed consent and then frozen in isopentane and liquid nitrogen. Frozen sections 6 μm thick were stained histochemically and immunologically. Activities of SDH and COX were visualized as described previously (Hasegawa et al., 1991; Dubowitz, 1985). The expression of MIDAS was detected with anti-MIDAS antibody. The polyclonal antibody against MIDAS was diluted 500-fold with 10% BSA in PBS and incubated with sections for 5 hours at 37°C and then MIDAS was detected with DAB using an indirect streptavidin-biotin immunohistochemical method, according to the manufacturer's protocol (Histofine, Nichirei, Co. Ltd., Tokyo, Japan). The MIDAS protein expressed was semi-quantified by the density of staining.

Immunocytostaining of cultured cells

Cultured cells were fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature. After a wash with PBS, they were treated with 5% acetic acid in ethanol for 10 minutes at –20°C to permeabilize membranes, then incubated in a blocking buffer (3% BSA and 3% goat serum in PBS) and overnight at 4°C in the blocking buffer containing primary antibody. After another wash with PBS, the cells were incubated in the blocking buffer containing labeled secondary antibody and visualized with a confocal laser-scanning microscope (Fluoview FV300, Olympus, Tokyo, Japan). As an alternative, we used another method described (Bell et al., 2001). In brief, cells were fixed for 10 minutes with 4% paraformaldehyde and 4% sucrose without treatment for permeabilization and incubated with primary antibody, followed by secondary antibody.

Subfractionation of HeLa cells

Cells were homogenized as described (Trounce et al., 1996). The homogenate was applied to a 7-35% (w/v) Nycodenz preformed continuous density gradient and centrifuged in a swinging-bucket rotor at 77,000 g_{AV} for 4 hours. The fractions were collected from the top of the gradient. The MIDAS protein was semi-quantified by the density of total bands in western blots. The sub-organellar fractionation of mitochondria (fraction number 15) was performed as described (Kanamori et al., 2003).

Electron microscopy

Cells were cultured on plastic dishes and fixed with 2% glutaraldehyde in PBS. Ultra-thin sections were stained with uranyl acetate and lead nitrate and examined with an H-7000 electron microscope (Hitachi, Tokyo, Japan).

Flow cytometry

Living transfectants were stained with 20 nM MitoTracker Red CMXRos (Molecular Probes) or 100 nM MitoTracker Green (Molecular Probes) for 30 minutes at 37°C, treated with trypsin and subjected to a flow cytometric analysis with an Epics Elite ESP (Coulter).