A Mutation in the SDHC Gene of Complex II Increases Oxidative Stress, Resulting in Apoptosis and Tumorigenesis

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Abstract

Intracellular oxidative stress from mitochondria is thought to be important in carcinogenesis and tumorigenesis, but direct experimental proof is limited. In this study, a transgenic mouse cell line (SDHC E69) with a mutated SDHC gene (a subunit of complex II in the electron transport chain) was constructed to test this question. The SDHC E69 cells overproduced superoxide anion (O2−) from mitochondria, had elevated cytoplasmic carbonyl proteins and 8-OH-deoxyguanine in their DNA as well as significantly higher mutation frequencies than wild type. There were many apoptotic cells in this cell line, as predicted by the observed increase in caspase 3 activity, decrease in mitochondrial membrane potential, and structural changes in their mitochondria. In addition, some cells that escaped apoptosis underwent transformation, as evidenced by the fact that SDHC E69 cells caused benign tumors when injected under the epithelium of nude mice. These results underscore the notion that mitochondrially generated oxidative stress can contribute to nuclear DNA damage, mutagenesis, and ultimately, tumorigenesis. (Cancer Res 2005; 65(1): 203-9)

Introduction

Much attention has focused on the role of oxidative stress in apoptosis and tumorigenesis (1–5). There is substantial experimental evidence directly supporting the idea that inactivation of antioxidant enzymes and their attendant oxidative stress are causal events in carcinogenesis and tumorigenesis. For example, Oberley et al. (6–8) proposed that superoxide anion (O2−), one type of reactive oxygen species (ROS), participates in the etiology of cancer, differentiation, and aging. We have previously isolated a mev-1(kn1) mutant of Caenorhabditis elegans, which has a genetic dysfunction in electron transport (9, 10). Consequently, this mutant overproduces O2− in its mitochondria (11). The mev-1 gene encodes Cyt-1, the cytochrome b560 large subunit of succinate-coenzyme Q oxidoreductase: complex II, which is homologous to SDHC in humans (10). The kn1 mutation leads to supernumerary apoptosis and precocious aging (12–14). Recently, it has been reported that mutations in the SDHC gene cause some familial chromaffin cell tumors (i.e., paragangliomas) in humans (15), but the mechanism by which these mutations cause cancer is still unknown. In order to determine the effects of mitochondrially derived ROS in mammals, we established a transgenic mouse fibroblast NIH3T3 cell line with the equivalent mutation in the SDHC gene as C. elegans mev-1.

As expected, complex II enzyme activity was diminished and O2− was elevated in the transgenic cell line, resulting in more cellular oxidative damage than in the isogenic wild-type cell line. The resultant oxidative stress induced many apoptotic cells. Moreover, the cells that escaped apoptosis were frequently transformed. These data show that ROS from mitochondria can promote not only apoptosis but also tumorigenesis, thus explaining the malignancies such as chromaffin cell tumors.

Materials and Methods

Predicted Amino Acid Sequences of SDHC Gene. The amino acid sequences of Escherichia coli (Genbank accession no. AE005250), C. elegans (Genbank accession no. L26545-1), Mus musculus (Genbank accession no. AK032458-1), Bos taurus (Genbank accession no. 574803-1) and Homo sapiens (Genbank accession no. D49737-1), and the sequences of complete cDNAs of E. coli (Genbank accession no. AE000175 or U00096), C. elegans (Genbank accession no. NM_066882), M. musculus (Genbank accession no. NM_025321), B. taurus (Genbank accession no. NM_175814), and H. sapiens (Genbank accession no. NM_033001) named SDHC, cyt-1 or cytobrome b large subunit were retrieved from DDBJ/GenBank/European Molecular Biology Laboratory and Entrez Nucleotide of National Center for Biotechnology Information, respectively.

Mouse SDHC cDNA Cloning. The DNA sequence of SDHC, a mouse homologue of C. elegans cyt-1 gene, was obtained from DDBJ (http://www.ddbj.nig.ac.jp/E-mail/homology.html). Wild-type SDHC PCR product was obtained from the templates of liver cDNA in an ICR mouse strain (primers 5’-GGGGAAATTCATGGGGTTCTTTGCTTGTAGACATGCAGCGG-3’ and 5’-GGGAAGCTTTCACAGGGCGGCCAGCCC-3’) and then was inserted in a pBluescript II SK+ vector at EcoRI-HindIII site.

Construction of a mev-1 Mutant-Type SDHC Gene and Establishment of the Mutant Cell Lines. The mutant allele was proliferated by PCR amplification (primers 5’-GGGGAAATTCATGGGGTTCTTTGCTTGTAGACATGCAGCGG-3’ and 5’-GGGAAGCTTTCACAGGGCGGCCAGCCC-3’). This 5’ primer contains the mutated nucleotide, and the PCR product was substituted at an Earl-HindIII site in the pBluescript II SK+ vector including the wild-type SDHC gene. This fragment was inserted in an expression vector containing basal transcription region of cytomegalovirus promoter (PmCMV), and the vector DNA was integrated in the chromosome of mouse embryonic fibroblast culture cells (NIH3T3 cells) using the LipofectAMINE plus reagent (Invitrogen, Inc., Tokyo, Japan).

Cell Culture. The cells were cultivated in DMEM medium (Nissui Co., Tokyo, Japan), including 2.5% fetal bovine serum + 2.5% calf serum in a 5% CO2 incubator. Cell division and proliferation were examined after synchronous culture in G0 phase into exhaustion of serum medium and at the contact inhibition state in 100-mm tissue culture dishes. For cell growth, 5 × 104 synchronized cells were cultured in a standard medium of 35-mm tissue culture dishes. These cells were stained by trypan blue solution and counted every 12 hours. For measurement of transformation rate, the cell culture method employed DMEM medium including 2.5% fetal bovine serum + 2.5% calf serum containing 0.33% soft agar (Difco Noble Agar) on a standard medium including 0.5% soft agar. Between 1–5 × 105 cells were cultured in the soft agar medium in 60-mm tissue culture dishes, and the number of colonies after 35 days was compared with that of 7 days.
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Biochemical Analyses. The activities of NADH-CoQ oxidoreductase (complex I) and complex II in mitochondria were measured as previously described (16).

O$_2$ Measurement. For mitochondrial isolation, living cells were obtained by trypsin treatment, collected by centrifugation, and homogenized (10% w/v) in isolation buffer [210 mmol/L mannitol, 70 mmol/L sucrose, 0.1 mmol/L EDTA and 5 mmol/L Tris-HCl (pH 7.4)] with a Teflon homogenizer. Mitochondria were isolated by differential centrifugation (16) and suspended in Tris-EDTA buffer [0.1 mmol/L EDTA and 50 mmol/L Tris-HCl (pH 7.4)]. O$_2$ production was measured using the chemiluminescent probe 2-methyl-6-p-methoxyphenylthethyl-imidazopyrazinone (ATTO, Tokyo, Japan). Forty micrograms of intact mitochondria were added to 1 mL of assay buffer [50 mmol/L HEPS-NaOH (pH 7.4) and 2 mmol/L EDTA] containing 0.7 pM 2-methyl-6-p-methoxyphenylthethyl-imidazopyrazinone. For the measurement of O$_2$ production from mitochondria with substrate and inhibitor, 1.5 mmol/L succinate as a complex II substrate were added to the mitochondrial solution, and 2 μg/mL of 2-heptyl-4-hydroxy-quinoline-N-oxide as a complex III inhibitor was added after addition of the mitochondria and succinate. These solutions were placed into the photon counter with an AB-2200 type Luminescencer-PSN (ATTO) and measured at 37°C. The rates of O$_2$ were expressed as counts per second and the amounts were calculated by subtracting the absorbance of samples in the presence of 10 μg/mL bovine Cu,Zn-superoxide dismutase from that in the absence of the enzyme.

Measurements of CPP32/Caspase 3, 8-OH-Deoxyguanine and Mutation Frequencies. CPP32/Caspase 3 activity was measured by DEVD (Asp-Val-Glu-Asp) (p-nitroanilide) substrate using CPP32/ Caspase 3 Colorimetric Protease Assay Kit (BV-K106, MBL, Nagoya, Japan) with a SpectraMax 250 (Molecular Devices Co., Synnyvale, CA). 8-OH-Deoxyguanine (8-OHdG) was measured by a direct immunofluorescence analysis (flow cytometry) using the FITC-conjugated antibody in a DNA Oxidative Damage Kit (DN-001, Kamiya Biomedical Co., Seattle, WA) with FACSscan (Becton Dickinson and Co., Lincoln Park, NJ). The cultured cells in a standard medium of 10-mm tissue culture dish were harvested for fixation into 2% paraformaldehyde and the dehydration using 70% ethanol. The 5 × 10$^6$ cells were incubated with FITC-conjugated antibody binds to 8-OHdG for 1 hour in dark at room temperature and the luminescence intensity by a flow cytometer was measured to quantify 8-OHdG. For measurement of mutation frequencies on the nuclear gene hprt, the 6-thioguanine (6-TG) tolerance test was done. Wild-type cells and 1-month SDHC E69 mutant cells were cultured in 6 μmol/L 6-TG containing medium and 3-month SDHC E69 mutant cells were cultured in 12 μmol/L 6-TG. The 6-TG concentrations were selected because they produce equal survival rates in the cell lines.

Slot Blot Analysis of Protein Carbonyls. Protein carbonyl accumulation was measured by using the anti-2,4-dinitrophenyl hydrazine antibodies; 0.5 mL of lysis buffer (10 mmol/L Tris-HCl, 100 mmol/L NaCl, 0.1% NP40, 1 mL EDTA, 0.1 mmol/L p-APMSF) were added in culture cells in a 100-mm tissue culture dish, and protein carbonyls contents of the extracts were determined by the 2,4-dinitrophenyl hydrazine method as described previously (17). The 2,4-dinitrophenyl hydrazine–treated cell extracts were measured protein carbonyls contents by slot blot analysis using MilliBlot-S (Millipore Co., Tokyo, Japan). The extractions were transferred to nitrocellulose membranes (Amersham Biosciences, Tokyo, Japan), and the membranes were treated with anti-2,4-dinitrophenyl hydrazine antibodies in TEN buffer (10 mmol/L Tris-HCl, 1 mL EDTA, 100 mmol/L NaCl) containing 5% skim milk. After washing with the buffer, the membranes were treated with an Enhanced Chemiluminescence Plus Western blotting detection system (Amersham Biosciences) after treated anti-rabbit antibodies and were exposed Hyperfilm enhanced chemiluminescence film (Amersham Biosciences) at room temperature for 2 minutes. The chemiluminescent signals were visualized in a CS Analyzer and AE-6962 light capture (ATTO).

Results and Discussion

We have established a mouse fibroblast NIH3T3 cell line that contains a missense mutation in SDHC, a subunit of complex II in electron transport. The resultant polypeptide possesses both the active site as well as the binding site to Heme and CoQ (Fig. 1A). The amino acid sequence at this region is highly conserved among animals (Fig. 1A; refs. 18–24). To construct a mutant SDHC transgene, the mouse SDHC gene was modified to convert valine to glutamic acid at position 69 (SDHC Val69Glu; Fig. 1B). It is thought that the serine residue within the CoQ binding region is an active center of oxidoreductase and acts as nucleophilic amino acid. This alternation would be predicted to reduce complex II activity. It might also result in an excess electron leakage from electron transport by decreasing the affinity between complex II and CoQ (20, 24) and thereby uncouple electron transfer. The mutation corresponds to the amino acid substitution of glutamic acid from glycine at position 71 (Gly71Glu) in the C. elegans mev-1 mutant allele knl (10). In addition, the mutant transgene contained a cutting site for Ncol, created by a thymine-to-cytosine substitution at position 192 (Fig. 1B). This enabled the mutant and wild-type alleles of the SDHC gene to be experimentally distinguished. This gene was transfected into mouse fibroblast NIH3T3 cells. From the transfected cells, we selected cell lines that expressed equal amounts of mRNA from the transgene and endogenous, wild-type allele (Fig. 1C). They were named SDHC E69. Knockout cell lines were not obtained, most likely because cells with more than 80% abnormal mitochondrial DNA are inviable (25). In addition, RNAi with cyt-1 produced an embryonic lethal in C. elegans (26). The enzymatic activity of complex II in SDHC E69 cells was reduced 40%, whereas the activity of complex I was unaffected in SDHC E69 cells (Fig. 1D). ATP levels were not affected (data not shown), suggesting that this mutation did not directly compromise cell survival through reduced respiration per se.

It is reasonable to assume that both the wild-type and mutant SDHC subunits competitively contributed to complex II in the mitochondria of SDHC E69 cells. Electron leakage would then be increased between complex II and CoQ by abnormal electron flow from the mutant SDHC. Of the two enzymatic functions associated with complex II (succinate-CoQ dehydrogenase and succinate-CoQ oxidoreductase), only succinate-CoQ oxidoreductase activity may be reduced. This supposition is based upon the observation that the C. elegans mev-1 mutant containing the same mutation in SDHC (Cyt-1) showed a reduction in succinate-CoQ oxidoreductase activity but not in either succinate-CoQ dehydrogenase activity (10) or ATP levels (11).

It is widely reported that the excess ROS cause apoptosis and carcinogenesis (1–5). With respect to the former, we found that the mev-1 mutant of C. elegans, which is the nematode equivalent to SDHC E69, is oxygen hypersensitive and contains supernumerary apoptotic cells due to the overproduction of O$_2$ from mitochondria (10–14). This suggested that apoptosis might also be caused by the oxidative stress from mitochondria in mouse SDHC E69 cells. We therefore examined O$_2$ production from the mitochondria isolated from mutant and wild-type cells. In wild-type cells, there was no difference in O$_2$ production when succinate was added as a substrate for complex II. In contrast, O$_2$ levels increased when the Complex III inhibitor 2-heptyl-4-hydroxy-quinoline-N-oxide was added to induce electron leakage from complex III (Fig. 2A). This is consistent with the notion that O$_2$ is not normally produced at complex II but is instead produced at complex III of the electron transport system. In the mutant cell line, O$_2$ production was slightly but not statistically significantly higher in untreated mitochondria. However, the addition of succinate to stimulate complex II activity resulted in a large increase in O$_2$ production in the mutant cell line.
Increased O$_2$ production is not a generalized feature of this cell line, because levels were somewhat lower than wild type when the inhibitor for complex III was added. The O$_2$ levels for both wild-type and SDHC E69 cells were not affected when NADH was added as a substrate of complex I (data not shown). In concert, these data suggest that, as with C. elegans, the elevated O$_2$ is generated at complex II in SDHC E69 cells. These in vitro data are consistent with observations made on superoxide anion levels in intact cells. Specifically, O$_2$ levels were significantly higher in intact mitochondria isolated from SDHC E69 cells 1 and 3 months after the establishment (Fig. 2B). This suggests that the mouse SDHC E69 cell line also suffers from mitochondrially produced oxidative stress.

It is known that oxidative stress can damage cellular components such as proteins and DNA (27–30). The SDHC E69 cells accumulated cytoplasmic carbonyl proteins, a marker of oxidative stress, at a faster rate than wild type (Fig. 2C). In addition, the amount of 8-OHdG, a DNA marker of oxidative stress (31) was 2-fold higher in SDHC E69 cells (Fig. 2D).

During 3 months (the time necessary for colony formation on the medium plates), wild-type NIH3T3 cells maintained normal fibroblast morphology and grew in a monolayer. Conversely, the SDHC E69 cells showed the loss of contact inhibition and had many apoptotic molecule-like granules during 1 month after establishment. During the period of colony formation, some clefts characteristics of programmed cell death (32, 33) were found in the center of some colonies (Fig. 3A). In 3-month SDHC E69 cells, the morphology was changed from the typical solid and elongated fibroblasts to smooth and rounded cells. Similar changes were evident, although to a lesser degree in the 1-month SDHC E69 cells. In addition, the SDHC E69 cells formed multiple layers (Fig. 3A). As shown in Fig. 3B, the doubling time of 1-month SDHC E69 cells was 1.5 to 2 times slower than that of wild-type cells (36–48 hours for 1-month SDHC E69 cells versus 20–24 hours for wild-type cells). However, the doubling time in 3-month SDHC E69 cells was completely recovered to that of wild type.

We have previously shown that the mev-1 mutation in C. elegans results in mitochondrial abnormalities that lead to reduced mitochondrial membrane potential (12). These pathologies likely explain the observation that mev-1 embryos had supernumerary apoptotic cells (12). We examined SDHC E69 cells for these same properties. First, electron micrographs revealed that many mitochondria in SDHC E69 cells had disorganized cristae. In addition, many mitochondria were swollen and enlarged (Fig. 4A). The distribution of mitochondria in wild-type and SDHC E69 cells was the same with most mitochondrial located around nuclei in both strains (Fig. 4B). We speculate that this enlargement in the SDHC E69 cells could have resulted from mitochondrial fusion to compensate
dysfunctional mitochondria (34–37). Second, the lipophilic cation JC-1 was employed to determine the level of phospholipids and membrane potential in SDHC E69 and wild-type cells. Excitation at 485 ± 11 nm provided an indication of phospholipid level, whereas excitation at 535 ± 17.5 nm allowed determination of relative membrane potential. SDHC E69 cells contained reduced amounts of phospholipids in the mitochondrial membrane and their membrane potential was significantly less than in wild type (Fig. 4C). Third, the activity of the apoptosis marker caspase 3 (38, 39) was 1.3 to 1.8 times higher in SDHC E69 cells (Fig. 4D). Caspase activation results in a series of events, including degradation of nuclear DNA into nucleosome-length pieces.

Figure 2. O$_2^-$ production from mitochondria and accumulation of 8-OHdG. WT, wild type. A. O$_2^-$ productions from mitochondria in vitro. *, P < 0.05. B. accumulation of O$_2^-$ in mitochondria in vivo. **: P < 0.01; ***: P = 0.067. C. Measurements of protein carbonyls by a Slot blot analysis using anti-DNPH antibodies. *, P < 0.01. D. Measurement of 8-OHdG by a direct immunofluorescence analysis using FITC-conjugated antibody binds to 8-OHdG.

Figure 3. Morphology and cell division and proliferation of SDHC E69 mutant cell lines. A, morphologies of the confluent wild-type cells (WT; for 3-month cultured cells) and 1- and 3-month SDHC E69 mutant cell lines (original magnification: top, ×100; bottom, ×40). There were no observable morphological changes in the wild-type cells during the 3-month culture period. White arrows, apoptotic molecule–like granules that resulted from apoptosis. B, cell division and proliferation of wild type (●), 1-month (●), and 3-month (▲) SDHC E69 mutant cell lines. *, P < 0.01.
fragments, that culminates in apoptosis (40, 41). DNA isolated from SDHC E69 cells also displayed the nucleosomal laddering characteristic of apoptosis (data not shown).

When SDHC E69 cells were injected under the epithelium of nude mice, 1-month cells rapidly disappeared as compared with those of wild type (Fig. 4E). This suggests that these cells were dying of apoptosis and were phagocytized shortly after injection. Conversely, injecting the same number of 3-month cells resulted in the production of tumors within 2 weeks (Fig. 4E). It is known that NIH3T3 cells are transformed at high frequency by spontaneous mutations during long passage time or culture time. In fact, the transformed cells grew into huge malignant tumors 1 month after
the injection under the epithelium of nude mice (data not shown). On the other hand, 3-month SDHC E69 cells transformed by intracutaneous oxidative stress grew two weeks on nude mice, but the size remained unchanged after 1 month (data not shown). These results suggest that transformed cells in 3-month SDHC E69 cells may develop into benign tumors rather than malignant tumors. This may be similar to the familial chromaffin cell tumors (i.e., paragangliomas) in humans that occur in individuals suffering mutations in the SDHC gene (15).

It is known that neoplastic transformation is the consequence of multiple mutations to oncogenes and tumor suppressor genes (28, 29, 42, 43). We examined the resistance to 6-TG as an indicator of mutations in the hprt gene (44, 45). The 3-month SDHC E69 cells were approximately twice more resistant than the 1-month SDHC E69 and wild-type cells (Fig. 4F). Under a 6- G concentration that allowed 2% survival during short-term culturing, only 3-month SDHC E69 cells had colony-forming ability (Fig. 4G). Most importantly, these data show that SDHC E69 cells were hypermutable. In addition, since tumor cells generally acquire anchorage-independent growth (46, 47), a soft agar culture method could not detect hypermutable SDHC E69 cells, which had colony-forming ability (Fig. 4G). These data imply that mitochondrially derived ROS can mutate other genes, including tumor suppressor genes and oncogenes, and can lead to transformation. This four steps sequence (mitochondrial ROS, nuclear DNA oxidative damage, mutation, and transformation) was shown in the current manuscript with SDHC E69 cells. Melov et al. (49, 50) have also shown the deleterious consequences of ROS generation in mitochondria. In particular, mice lacking the mitochondrial superoxide dismutase were shown to have elevated levels of oxidative damage to their nuclear DNA. This same mitochondrial defect results in a 9-fold increase in chromosome rearrangements. Thus, mitochondrial dysfunction can lead to DNA damage and mutation and those events can ultimately lead to apoptosis and transformation. In addition, Oberley et al. (6–8) argued the importance of O2− in cancer, differentiation and aging. In humans, a mutation in complex II was found in patients of some familial chromaffin cell tumors (i.e., paragangliomas; ref. 15). Our mouse system now provides an explanation for the mechanism by which this mitochondrial dysfunction causes tumorigenesis.

We have shown that the damage induced by oxidative stress from mitochondrial dysfunction can lead to apoptosis and tumorigenesis. However, these may ultimately play roles in different diseases in addition to neoplasms. Specifically, whereas apoptosis may have evolved as a defense system to remove cells damaged by oxidative stress, excessive apoptosis may result in neuronal degeneration and aging (51–54).

In conclusion, we succeeded in establishing SDHC E69 cell lines with a single amino acid substitution in SDHC gene. The study of these cell lines provided direct evidence that O2− production from mitochondria results in oxidative stress. Moreover, this oxidative stress leads to pathologies such as apoptosis, precocious aging and neuronal degeneration, and tumorigenesis. Thus, we have provided linkage between excess O2− production from mitochondria, intracellular oxidative stresses, and tumorigenesis.

Acknowledgments

Received 7/29/2004; revised 10/2/2004; accepted 10/22/2004.

Grant support: Virtual Research Institute of Aging of Nippon Boehringer Ingelheim, Sumitomo Foundation, Takeda Science Foundation, and grant-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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We thank Dr. O. Niwa, Radiation Biology Center, Kyoto University, for kind advice of mutation experiments; H. Mitani and Y. Hirayama, Cancer Institute, Japanese Foundation for Cancer Research, for the work using nude mice; and Drs. S. Goto and R. Takahashi for anti-2,4-dinitrophenyl hydrazine antibodies against protein carbonyls.

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