Mitochondrial impairment in p53-deficient human cancer cells

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The mechanism linking p53 inactivation to human cell malignancy remains unclear. Studies have indicated that mitochondrial dysfunction is involved in carcinogenesis. In this study we investigated the role of p53 in mitochondrial DNA (mtDNA) mutation and maintenance of proper mitochondrial function. We measured mtDNA mutation and found no difference in frequency of mutation between the p53+/+ and p53−/− cell lines. However, mitochondrial cytochrome c oxidase (COX) activity was significantly diminished in p53−/− cells. This decrease in COX activity was attributed to decreased protein levels of the COXII subunit encoded by the mitochondrial genome and was not due to mutation in the mitochondrial COXII gene. Further investigation revealed no concomitant decrease in COXII mRNA levels in p53−/− cells and the stability of mRNA in p53−/− cells was unaffected. This study suggests that decreased COX activity is likely due to post-transcriptional regulation of the COXII subunit by p53. COX is a critical enzyme in the mitochondrial electron transport chain and reduced COX activity may affect mitochondrial structure. However, examination of mitochondrial ultrastructure revealed no obvious differences between p53+/+ and p53−/− cell lines. Together, our study suggests that p53 is involved in regulation of COXII at the protein level but not at the mRNA level. p53 does not affect mtDNA mutation or mitochondrial ultrastructure.

Introduction

Mitochondrial dysfunction is a hallmark of cancer cells. Several notable differences between the mitochondria of normal versus transformed cells have been reported (reviewed in Chang et al., 1971; Pedersen, 1978; Carafoli, 1980; Singh, 1998, 1999). For example, various tumor cells exhibit differences in the number, size and shape of their mitochondria relative to normal cells. The mitochondria of rapidly growing tumors tend to be fewer in number, smaller and have fewer cristae than mitochondria from slowly growing tumors that are larger and have characteristics more closely resembling those of normal cells. In addition, the ultrastructural features of mitochondria in oncocytesoma of thyroid, salivary gland, kidney, parathyroid and breast cells show features in common with mitochondrial encephalomyopathies, where mitochondria are found as large aggregates and display a variety of morphological alterations (Maximo and Sobrinho-Simoes, 2000). Mitochondrial DNA (mtDNA) mutations are also commonly found in many tumors (Polyak et al., 1998; Singh, 1998; Fliss et al., 2000; Penta et al., 2001; Modica-Napolitano and Singh, 1996, 2002).

p53 is a multifunctional protein. Most of the cellular functions of p53 have been described to be in the nucleus. However, recent studies indicate that p53 is also localized in mitochondria (Katsumoto et al., 1995; Merrick et al., 1996; Marchenko et al., 2000; Donahue et al., 2001). Several lines of evidence suggest that p53 and mitochondrial function are interconnected. One pertains to the integral role of p53 in regulating mitochondrial membrane potential (Li et al., 1999). It is demonstrated that p53−/− mice are resistant to apoptosis induced through a collapse of mitochondrial membrane potential. It is suggested that p53 mediates apoptosis through a three step process: (i) transcriptional induction of redox-related genes; (ii) formation of reactive oxygen species; (iii) oxidative degradation of mitochondrial components (Polyak et al., 1997). Several genes whose products localize to mitochondria contain a p53 regulatory element (Miyashita and Reed, 1995; Bourdon et al., 1997). Mitochondrial localization of p53 has also been correlated with early changes leading to p53-mediated apoptosis in different cell types induced by a range of apoptosis inducers, including DNA damage and hypoxia (Marchenko et al., 2000; Donahue et al., 2001). Mouse embryos deficient in p53 protein show a deficiency of 16S rRNA transcript encoded by mtDNA (Ibrahim et al., 1998).

In the nucleus, p53 plays an important role in DNA base excision repair, both as a transcription factor and as a component of nucleotide and base excision repair complexes (Offer et al., 1999; Morris, 2002). p53 interacts with RAD51 and topoisomerases I and II. Each of these proteins is involved in recombination. Consistent with this finding, an increased rate of recombination has been reported in p53-deficient cells (Buchop et al., 1997; Yuwen et al., 1997; Gobert et al., 1999; Dudenhofer et al., 1999). Loss of p53 protein and mitochondrial function has been extensively reported in a variety of cancers (Nigro et al., 1989; Baker et al., 1990; Auer et al., 1994; Levine, 1997; Polyak et al., 1998; Singh, 1998). However, it is not clear whether p53 is involved in mtDNA mutagenesis and in maintaining proper mitochondrial function. The purpose of the present study was to investigate the effect of p53 inactivation on mitochondrial genetic response and changes in mitochondrial function.

Materials and methods

Cell culture and colony formation assays

Human colon cancer cell line HCT116 (p53+/+) and its derivative HCTp53KO (p53−/−), which has both p53 alleles disrupted (Bunz et al., 1998), were grown in McCoy’s 5A medium supplemented with 10% fetal bovine serum and penicillin/streptomycin, in a humidified incubator at 37°C, 5% CO2. For colony formation assay, 100 cells were seeded in 60 mm tissue culture dishes containing 3 ml of fresh medium supplemented with varied concentrations of chloramphenicol (CAP) and cultured for 6 days. Surviving colonies were fixed/stained and counted. All dose points were measured in duplicate in each experiment and survival curves represent data from two independent experiments.

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Mitochondrial enzyme activity assays

Activities of mitochondrial enzymes were determined spectrophotometrically according to previous methods with slight modifications (Singer, 1994; Trounce et al., 1996). Cells were trypsinized and washed with phosphate-buffered saline and then resuspended in the assay buffer. Cell membranes were permeabilized with 0.5% Triton X-100. Samples were kept on ice for subsequent determination of mitochondrial enzyme activities. Specifically, Complex I (NADH dehydrogenase) activity was measured by monitoring the reduction of ferricyanide at 420 nm (Singer, 1994). Brieﬂy, to 1 ml of 120 mM triethanolamine buffer, pH 7.8, containing 0.5 mM ferricyanide and 5 mM rotenone, cells (~80 µg) were added to start the reaction. The NADH-dependent reduction of ferricyanide was measured at 30°C for 2 min. The activities are expressed as nmol reduced ferricyanide/min/mg protein, using an extinction coefﬁcient of 1.0 per mM/cm. Complex II (succinate dehydrogenase) activity was measured as the rate of reduction of 2,6-dichlorophenolidophenol (DCPIP) at 600 nm, when coupled to the complex II-catalyzed reduction of decylubiquinone (DB) (Trounce et al., 1996). Brieﬂy, cells (~80 µg) were added to 1 ml of 0.1 M potassium phosphate buffer, pH 7.4, containing 20 mM succinate. The mixture was incubated at 30°C for 5 min followed by addition of antimycin (2 µg), rotenone (2 µg), KCN (2 mM) and DCPIP (50 µM). The reaction was started by addition of 50 µM DB. The activities are expressed as nmol DCPIP/min/mg protein, using an extinction coefﬁcient of 19.1 per mM/cm. Complex III (ubiquinone–cytochrome c oxidoreductase) activity was measured by monitoring reduction of cytochrome c at 550 nm catalyzed by complex III in the presence of reduced decylubiquinone (DBH2) (Trounce et al., 1996). Cells were added to 1 ml of Tris–succrose buffer (50 mM Tris, 250 mM sucrose, 1 mM EDTA, pH 7.4), containing 50 mM cytochrome c and 2 mM KCN. The mixture was incubated at 30°C for 5 min. The reaction was started by adding 50 mM DBH2 and measured for 2 min. The activities are expressed as nmol cytochrome reduced/min/mg protein with an extinction coefﬁcient of 19.0 per mM/cm. Complex IV (cytochrome c oxidase) activity was measured by monitoring the oxidation of reduced cytochrome c at 550 nm (Trounce et al., 1996). Brieﬂy, to 1 ml of 20 mM potassium phosphate, pH 7.4, containing 20 mM succinate, cells (~80 µg) were added to start the reaction. Readings were recorded for 2 min. The activities are expressed as nmol cytochrome oxidized/min/mg protein with an extinction coefﬁcient of 19.0 per mM/cm.

RT–PCR analysis

Levels of COXI, COXII and COXIII mRNA were evaluated by RT–PCR. Total RNA was isolated using TRIzol reagent. For the analysis of mRNA stability, cells were incubated with 5 µg/ml actinomycin D and RNA was isolated at various times thereafter. RNA was annealed to oligo(dT) primers at 70°C and reverse transcribed with SuperScript II RNase H– reverse transcriptase (Gibco BRL) at 42°C in the presence of dithiothreitol and 200 µM each dNTP. Primer sequences were as follows: COXI forward primer, 5′-ATGTTCGCCGACCGTTGACT-3′; reverse primer, 5′-GTATCACGGTTCTTC-3′; COXII forward primer, 5′-ATGCCACATCGACGC-3′; reverse primer, 5′-CTATAGGGTAAATACCCGGCC-3′; COXIII forward primer, 5′-ATGACCCATACATACATGC-3′; reverse primer, 5′-AGACCCCTCATATAGG-3′. PCR was initially performed with different primer concentrations to ﬁnd the optimal number and sample dilution for quantitative ampliﬁcation of target and control genes. Subsequently, PCR was performed for 20 cycles, which was optimal for a linear response. GPIHPR served as a control. PCR products were resolved on a 1.0% agarose gel and stained with ethidium bromide. The gel was photographed with Polaroid ﬁlm.

Western blot analysis

The expression of COXI and COXII was assessed by western blotting analysis of total protein lysate. Cells at ~75% conﬂuence were homogenized in 1 ml of homogenization buffer (20 mM Tris–HCl, pH 7.5, 5 mM MgCl2, 0.1 mM phenylmethylsulfonyl ﬂuoride, 20 µM leupeptin, 20 µM aprotinin) (Takahashi et al., 1995). The protein concentrations were determined using the Bio-Rad protein assay with BSA as standard. Ten micrograms of each sample was loaded onto 12% SDS–polyacrylamide gels and electrophoresed to resolve proteins. The proteins were then transferred onto nitrocellulose membranes. Membranes were blocked in 20 mM Tris–HCl, pH 7.5, 150 mM NaCl and 5% non-fat dry milk. Membranes were ﬁrst probed with anti-COXI and anti-COXII antibodies (Molecular Probes, Eugene, OR) and then with peroxidase-conjugated rabbit anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA). COXI and COXII were visualized with ECL detection reagents (Amersham Life Science, Little Chalfont, UK).

Cell proliferation assay

Cell proliferation was spectrophotometrically evaluated by the MTT reduction assay as described previously (Mossmann, 1983). Brieﬂy, cells were seeded at 1×105 cells/well in 24-well plates. Surviving cells were stained with MTT at days 0, 1, 2 and 3. The absorbance was determined at 553 nm.

Results

mtDNA mutational analysis

We investigated whether p53−/− cells accumulated spontaneous point mutations in the mitochondrial genome. One of the readily obtainable genetic markers for point mutation in the mitochondrial genome is resistance to the antibiotic CAP. CAP inhibits mitochondrial protein synthesis selectively by binding to the peptidyltransferase domain of 16S rRNA in mammalian cells (Branlant et al., 1981; Hashiguchi and Ikushima, 1998). Resistance to CAP arises by single base substitution(s) in the 16S rRNA gene encoded by the mitochondrial genome (Blanc et al., 1981; Kearsey and Craig, 1981; Howell and Kubacka, 1993). We treated both p53+/− and p53−/− cell lines with varied concentrations of CAP. Figure 1 demonstrates that there was no difference in number of CAP-resistant colonies between the two cell lines. This indicates that there is no difference in frequency of spontaneous mtDNA mutations between the p53+/− and p53−/− cell lines. In order to identify mutations in other regions of the mitochondrial genome, we PCR ampliﬁed genes encoding cytochrome c oxidase subunits I, II and III and found no changes in sequence of these genes between the wild-type cells and cells deﬁcient in p53 function (data not shown). We conclude that p53 does not play a role in mtDNA mutagenesis.
for three to four complexes in p53 enzyme activity. We compared activities of the mitochondrial III activity. (P. analysis. The decrease compared with the wild-type, using Student’s t-test for data analysis. The P value for (D) is 0.004, for (A)–(C) >0.05 (no statistical difference).

Decreased cytochrome c oxidase activity in p53 KO cells
p53 regulatory elements are found in the upstream promoter region of many genes whose products play important roles in mitochondrial metabolism (Miyashita and Reed, 1995; Bourdon et al., 1997). In the present study we determined whether p53 inactivation could lead to altered mitochondrial enzyme activity. We compared activities of the mitochondrial complexes in p53 KO and p53 KO cells. As shown in Figure 2, there were no differences in activities of complexes I, II and III between the two cell lines. However, complex IV activity in p53 KO cells was significantly lower compared with p53 KO cells, with an activity of 1.88 ± 0.29 in p53 KO and 1.27 ± 0.33 in p53 KO cells (~30% lower) (Figure 2). This study suggests that p53 inactivation leads to reduced complex IV activity.

Decreased level of COX II protein in p53 KO cells
Subunits of complex IV are encoded by both the nuclear and mitochondrial genomes. We investigated whether the alteration in complex IV activity was due to lower levels of cytochrome c oxidase protein encoded by the mitochondrial genome. Therefore, we examined the expression of COXI, COXII and COXIII mRNA by RT–PCR. In this experiment, G3PDH was used as the control. The results show no differences in the levels of COXI, COXII and COXIII mRNA between p53 KO and p53 KO cells (Figure 3). We next examined the protein levels of COXI and COXII by western blot analysis using antibodies against COXI and COXII. The same blot was probed with an antibody against actin as a control for variations in loading. The results show that the COXII protein level was significantly reduced in p53 KO cells (Figure 4B), while no change was observed in COXI (Figure 4A). Since COXII protein levels differed, we investigated whether the diminished COXII protein level in p53 KO cells is attributable to a decreased stability of mRNA. We treated cells with 5 µg/ml actinomycin D, an inhibitor of mRNA synthesis. As shown in Figure 5, there was no difference in the time-dependent decrease in mRNA level between p53-proficient and p53-deficient cells. The mRNA level was equally diminished even after 20 h treatment with actinomycin D. Taken together, the discordant expression of COXII mRNA and COXII protein suggest post-transcriptional regulation of COXII by p53.

Mitochondrial ultrastructure in p53-deficient cells
Since the complex IV activity was diminished in p53 KO cells, we determined whether this alteration would change the ultrastructure of mitochondria. Electron microscopy was conducted as described in Materials and methods. Figure 6A and B shows that both p53 KO and p53 KO cells contain normal mitochondrial structure with well-organized cristae being present in both cell lines. Furthermore, no difference in cell proliferation was observed when these two cell lines were grown in medium containing galactose (data not shown). Functional mitochondria are required for galactose utilization and a difference in proliferation would reflect a defect in mitochondrial function (Robinson et al., 1992). These studies suggest that inactivation of p53 does not result in significant changes in mitochondrial structure and does not affect proliferation.

Discussion
The results of the present study suggest that p53 is involved in regulation of mitochondrially encoded cytochrome c oxidase II. COX activity is described to be lower in colonic adenocarcinoma and hepatocellular carcinoma than in the normal cells (Sun and Cederbaum, 1980; Sun et al., 1981; Heerdt et al., 1990). COX deficiency is also one of the most frequent
Fig. 4. Western blot analysis of COXI and COXII gene expression. The protein lysates were prepared as described in Materials and methods. Ten milligrams of sample was loaded in each lane onto 12% SDS-polyacrylamide gels and electrophoresed to resolve proteins. Western blot analyses were performed using anti-COXI and anti-COXII antibodies and peroxidase-conjugated rabbit anti-mouse IgG. COXI and COXII were visualized with ECL detection reagents. (A) Expression of COXI. (B) Expression of COXII. (C) Quantitation of COXII against actin expression. The mean intensities are 0.78 ± 0.24 in p53+/+ and 0.32 ± 0.17 in p53−/− cells and P = 0.037, indicating a statistical difference between the wild-type p53+/+ and p53−/−.

Fig. 5. Analysis of COXII mRNA stability. The stability of COXII mRNA was analyzed by RT–PCR. Cells were incubated with 5 µg/ml actinomycin D and total RNA was isolated at various times thereafter. RT–PCR was performed as described in Materials and methods. 0, 2, 4, 6 and 20 h indicates time after treatment of cells with 5 µg/ml actinomycin D.

causes of respiratory chain defects in humans. Patients with COX deficiency present heterogeneous clinical phenotypes, including hepatic failure and encephalomyopathy (Barrientos et al., 2002). Although our analysis showed a decrease in COXII subunit protein, we did not find a corresponding decrease in COXII mRNA in p53−/− cells. It is possible that

Fig. 6. Mitochondrial ultrastructure in p53+/+ and p53−/− cells. (A) Ultrastructure of p53+/+ cell. (B) Ultrastructure of p53−/− cell. The protocol used to carry out microscopy is described in Materials and methods.
We investigated whether genes encoding COXI, COXII and p53 expression reduces the frequency of point mutations in a mitochondrial genome (Donahue et al., 2001). Together, our study suggests that COXII is post-transcriptionally regulated by p53 and that the lower level of COXII contributes to the reduced COX activity in p53−/− cells. Consistent with our results in human cells, Saccharomyces cerevisiae COXII is also regulated at the post-transcriptional level (Pinkham et al., 1994).

Mitochondrial mutations and structural abnormalities in mitochondria are associated with various tumors (Cavalli and Liang, 1998; Polyak et al., 1998; Fliss et al., 2000; Yeh et al., 2000). Studies also indicate that p53 may be intimately involved in biogenesis and proper mitochondrial function (Polyak et al., 1997; Giaccia and Kastan, 1998; Ibrahim et al., 1998; Prives and Hall, 1999; Donahue et al., 2001). However, our studies revealed no significant differences in ultrastructure of mitochondria or deficiency in growth of p53−/− deficient cells when grown in medium containing galactose (data not shown). It is noteworthy that galactose is utilized via mitochondrial metabolism. So if cells contain defective mitochondria they will grow slower than cells with intact mitochondria (Robinson et al., 1992).

p53 maintains the stability of the nuclear genome. Recent evidence suggests that p53 is involved in base excision repair (Zhou et al., 2001), nucleotide excision repair (Hwang et al., 1999) and recombination (Mkeel et al., 1997) in the nucleus. p53 expression reduces the frequency of point mutations in a shuttle vector modified by several chemical mutagens (Courtemanche and Anderson, 1999). Mutations in genes encoded by mtDNA have been described in carcinoma cells (Polyak et al., 1998). Homoplasmic mutations in mtDNA are also reported in tumors harboring p53 mutations (Polyak et al., 1998; Fliss et al., 2000). It is not clear whether p53 is involved in maintaining the integrity of the mitochondrial genome. We investigated whether genes encoding COXI, COXII and COXIII are mutated in p53−/− cells. Our analysis revealed no differences between the p53+/+ and p53−/− coding sequences of the COXI, COXII and COXIII genes. In addition, we measured the frequency of mitochondrial mutation in the 16S rRNA gene by determining chloramphenicol-resistant colonies in wild-type and p53-null mutants cells. We did not find a significant difference in the frequency of mutation in the 16S rRNA gene encoded by mtDNA. These studies suggest that p53 does not play a significant role in maintaining the integrity of the mitochondrial genome in human cancer cells. We conclude that mtDNA mutations and mitochondrial dysfunction are not ascribed to defects in p53 and that another gene(s) may be the central mediator of mitochondrial mutations and mitochondrial dysfunction in cancer cells.

Our data suggest that p53 protein specifically affects COXII expression at the protein level but not at the mRNA level. The results presented in this paper point to a novel mechanism by which p53 may regulate mitochondrial gene expression. However, further work is needed to elucidate the exact mechanism by which p53 brings about this effect in the mitochondria.

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