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A Method for Assessing Damage to Mitochondrial DNA Caused by Radiation and Epichlorohydrin

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SUMMARY

This paper describes a rapid and reliable method for quantification of damage to mitochondrial DNA (mtDNA), especially strand breaks. The degree of damage to mtDNA is assessed by the proportion of physical forms (i.e., supercoiled versus open-circular and linear forms) upon agarose gel electrophoresis, blotting, and visualization by hybridization with [^{32}P]mtDNA probes. The use of a radiolabeled probe is a crucial step in the procedure because it provides both a means to quantify by radioautography and to obtain the mtDNA specificity required to eliminate misinterpretation due to nuclear DNA contamination. To demonstrate the utility of this technique, X-irradiation and epichlorohydrin are shown to damage both isolated mtDNA and mtDNA in whole cells in a dose-dependent fashion.

INTRODUCTION

Mitochondrial DNA comprises about 0.1–1% of the total DNA in most mammalian cells. It codes in part for several critical mitochondrial inner membrane proteins including subunits of cytochrome oxidase and ATPase (1) and has been entirely sequenced for human (2), mouse (3), and cow (4). Since damaged mtDNA[3] appears to be repaired poorly (5, 6) and is not protected by histones (7), we are exploring mtDNA as a potential critical target of cytotoxic agents. There is reason to explore this hypothesis because it has been shown that, per nucleotide, mtDNA is 5- to 500-fold more sensitive than nuclear DNA to covalent binding by several chemicals, the highest reported differential being that for polycyclic aromatic hydrocarbons (8).

mtDNA in animals cells can be isolated primarily as a supercoiled, circular molecule (Fm I) (9) and Fm I molecules containing a short single strand which is base paired to a specific region of the genome, thus creating a relaxed, partially covalently closed circle with a short triple-stranded region termed the D-loop (10, 11). These forms migrate almost identically on ethidium bromide-containing agarose gels or cesium chloride gradients presumably because both are converted to tightly supercoiled molecules by dye intercalation. Introduction of one or more nicks in either intact strand of such molecules produces relaxed circular molecules (Fm II) which migrate distinctly on gels or gradients. Linear molecules (Fm III) may result from either extensive single strand breaks which create nicks on opposite strands at nearly the same position, or direct double strand breaks. In addition to these forms, variable and usually minor amounts of dimeric and replicative forms may be seen (6). Therefore any chemical or physical treatment that introduces strand interruptions can be monitored directly by conversion of Fm I to Fm II molecules. Finally, in order to ensure the identification of mtDNA, we have incorporated the use of specific mtDNA hybridization probes to Southern blots (12) of agarose gels. In this study, we report a test of the method by monitoring damage to isolated mtDNA and mtDNA in cell culture caused by irradiation or by epicholorohydrin.

MATERIALS AND METHODS

Isolation of mtDNA. mtDNA was isolated from mouse liver or HeLa cells. C57BL/6J male mice (25 g) were used. Animals were killed by decapitation, and livers were removed and rinsed. HeLa cells were grown in suspension in fortified minimum essential Eagle's medium with Hanks' salts and 5% fetal calf serum. The tissue was homogenized in MSB-Ca^{2+} buffer (containing 0.2 M mannitol, 0.07 M sucrose, 0.05 M Tris, and 0.03 M $CaCl_2$ at pH 7.5). Mitochondria were prepared by differential centrifugation and lysed with sodium dodecyl sulfate (1% final concentration), and proteins were removed by 50:50 phenol/chloroform extraction. DNA was precipitated with 95% ethanol in 0.3 M sodium acetate (final concentration), washed with 70% ethanol, vacuum dried, and resuspended for further study.

Treatment. Isolated mouse liver mtDNA or intact HeLa cells were irradiated on ice using a ^{137}Cs source (Mark I irradiator, J. L. Sheppard and Associates). Mouse liver mtDNA was exposed to epichlorohydrin

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[3] The abbreviation used is: mtDNA, mitochondrial DNA.

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for 15 minutes at 4°. Epicholorohydrin (Aldrich) was diluted in ethanol. Control mtDNA was exposed to ethanol for the same time period (15 min) as the chemical.

Agarose gel electrophoresis. Gels were prepared using 0.7% agarose in Tris-borate-EDTA, pH 8.0, containing 2 μg/ml of ethidium bromide. The samples were run for 2 hr at 100 V. Most of the nuclear DNA was retained on the top of the gel whereas mitochondrial DNA entered the gel. The gels were then soaked in 0.5 M NaOH containing 1.5 M NaCl for 1 hr to denature the DNA. One hour before blotting, the gel was neutralized with 1 M Tris in 1.5 M NaCl. Southern blotting (12) was performed with nitrocellulose paper. The filters were then hybridized for 24 hr with specific probes which detect only mitochondrial DNA.

Preparation of hybridization probes. Cloned mtDNA from bovine liver was prepared as described by Hauswirth and Laipis (13). mtDNA was cut by *Eco*RI and the largest fragment (9 kilobases) was cloned in plasmid pACYC 184. Plasmid DNA was isolated from 2-liter cultures of *Escherichia coli* HB 101 by the method of Birnboim and Doly (14). This DNA containing the bovine mtDNA insert was labeled with ^{32}P-nucleotides by nick translation (13). Because the bovine mtDNA is about 70% homologous in sequence to mouse and human mtDNA (2), it remains a sensitive and specific probe for hybridization to mouse and human mtDNA. Although pACYC 184 sequences are present in the probe, they do not hybridize to cellular DNA under these conditions.[4]

Quantitation of mtDNA. The blots were exposed to X-ray film (Kodak XAR-5) in a Kodak X-Omatic cassette with intensifying screens. A densitometric comparison of the autoradiographic band intensities was made using an E-C Apparatus Corp. microdensitometer. Band areas were determined at low to moderate autoradiographic exposures so that the band intensities remained in the approximately linear response range of the film.

RESULTS

Damage caused in isolated mtDNA by irradiation. Figure 1 depicts a [^{32}P]mtDNA-hybridized Southern blot (upper panel) of untreated and X-irradiated and/or heat-denatured DNA after electrophoresis on agarose in a system containing ethidium bromide. In control, untreated preparations of mtDNA (lanes *1* and *2*), about 90% of the mtDNA was found in Fm I and about 10% in Fm II. Fm I mtDNA is known to migrate faster than Fm II. The existence of Fm II reflects a combination of the intracellular fraction of Fm II and damage during isolation. Isolated mtDNA exposed to increasing amounts of X-irradiation exhibited a dose-related decrease in Fm I and an increase in Fm II molecules (lanes *5*, *6*, and *7*). Although an equal amount of mtDNA was used at each X-ray exposure, progressively less mtDNA appeared in Fms I and II with increasing exposure. Therefore, the fraction of Fm I [expressed as area Fm I/(area Fm I + area Fm II)] was used as a measure of damage rather than the absolute area of Fm I. Reasons for the lack of total recovery of mtDNA and alternative ways to quantitate damage are discussed below. The fraction of Fm I mtDNA is inversely related to the X-ray dose; Fm I/(Fm I + Fm II) decreased from 0.91 to 0.76, 0.60, or 0.16 upon exposure to 2,500, 5,000, or 20,000 rads, respectively. This experiment was done three times. In controls, the percentage of isolated mtDNA detected as Fm I varied from 70 to 91; regardless of this initial distribution, the percentage of Fm I converted to Fm II by a given dose of radiation varied by less than 5% in the different experiments. The conversion of Fm I to Fm II by radiation was significantly different from controls ($p < 0.05$).

To substantiate the identification of Fm I and Fm II, untreated (lanes *3* and *4*) or X-irradiated (lanes *8*, *9*, and *10*) mtDNA was heated to 90° for 5 min and then cooled before electrophoresis. The mtDNA that had been Fm II now migrated faster than Fm I, the mobility of which was unaffected by heat treatment. This presumably reflects the inability of heat-denatured strands that constitute Fm II to re-anneal after rapid cooling. The rapidly migrating derivative(s) from Fm II probably represents single-stranded circular and linear DNAs resulting from Fm II molecules containing a nick(s) on one or both of its two strands.

Figure 1 also depicts for comparison the ethidium bromide fluorescence (lower panel) of the agarose gel from which the Southern blot was prepared. Despite mobilities similar to those of Fm I and II, the fluorescent ethidium bromide-enriched bands are distinct from those containing mtDNA identified by [^{32}P]mtDNA-hybridized Southern blots. The fluorescent bands are not likely to be circular duplex DNA molecules because they disappear upon heat denaturation. Futhermore, they are not affected by X-irradiation. The lack of clear identification of mtDNA as fluorescent bands presumably reflects the low concentration of these molecules relative to contaminant nucleic acids. Treatment with RNase did not eliminate the major fluorescent band in the lower panel of Fig. 1.

To demonstrate the general utility of this technique, similar experiments were performed using epichlorohydrin in place of X-irradiation. Epichlorohydrin was chosen because its toxicity is known not to require metabolic activation, and it causes DNA strand breaks. Again, a dose-dependent response was seen. Exposure of mtDNA to 0.25% (25 mM) epichlorohydrin caused the fraction of Fm I [i.e., Fm I/(Fm I + Fm II)] to decrease from 0.84 (control) to 0.53 (mean of three experiments). Exposure to 25% (2.5 M) epichlorohydrin caused a further decrease in the fraction of Fm I to 0.16. Thus, the effect of both ionizing radiation and cytotoxic chemicals on mtDNA integrity can be monitored sensitively.

Damage of HeLa cell mtDNA by X-irradiation. When intact cells were X-irradiated, there was rapid conversion of Fm I to Fm II mtDNA. The damage was once again dose related (Fig. 2). Approximately twice as much X-irradiation was required to cause the same extent of damage in cells relative to isolated mtDNA. However, recovery of the sum of Fm I and Fm II was approximately the same for control cells and for cells irradiated with different doses of radiation. The area under the densitometric curves for Fm I/Fm II for control cells was 1451:364, and for cells X-irradiated with 5,000, 20,000, and 40,000 rads was 1235:424, 624:1112, and 336:1597, respectively. Thus, the assay also specifically detected mtDNA damage resulting from strand scission events in intact cells. This is a critical extension of the technique which allows evaluation of mtDNA as a potential target for a wide variety of cytotoxic drugs.

[4] W. W. Hauswirth, unpublished.