Singlet Oxygen Mediates the UVA-induced Generation of the Photoaging-associated Mitochondrial Common Deletion*

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Mutations of mitochondrial (mt) DNA accumulate during normal aging. The most frequent mutation is a 4,977-base pair deletion also called the common deletion, which is increased in photoaged skin. Oxidative stress may play a major role in the generation of large scale mtDNA deletions, but direct proof for this has been elusive. We therefore assessed whether the common deletion can be generated in vitro through UV irradiation and whether reactive oxygen species are involved in this process. Normal human fibroblasts were repetitively exposed to sublethal doses of UVA radiation and assayed for the common deletion employing a semiquantitative polymerase chain reaction technique. There was a time/dose-dependent generation of the common deletion, attributable to the generation of singlet oxygen, since the common deletion was diminished when irradiating in the presence of singlet oxygen quenchers, but increased when enhancing singlet oxygen half-life by deuterium oxide. The induction of the common deletion by UVA irradiation was mimicked by treatment of unirradiated cells with singlet oxygen produced by the thermodecomposition of an endoperoxide. These studies provide evidence for the involvement of reactive oxygen species in the generation of aging-associated mtDNA lesions in human cells and indicate a previously unrecognized role of singlet oxygen in photoaging of human skin.

Oxidative phosphorylation in mitochondria is carried out by five protein complexes encoded by both the nuclear DNA and the mitochondrion’s own genome, the mitochondrial (mt) DNA. Mutations of mtDNA have been shown previously to play a role in a variety of degenerative diseases mainly affecting muscle and nerve tissues (1–3) as well as diseases such as familial diabetes mellitus (4). Their relevance is not restricted to degenerative diseases, however; e.g. mtDNA mutations are also critically involved in the normal aging process (5–8).

The most frequent and best characterized mutation in mtDNA is a deletion of 4,977 base pairs in length, also called the common deletion. This common deletion is considered to be a marker for mutations in the mitochondrial genome, and substantial efforts have been made to elucidate the mechanism by which it is generated. A modified slip-replication mechanism has been proposed (9–12) involving the misannealing of direct repeats (Scheme 1). Hotspots for the common deletion exhibit structural abnormalities facilitating the misannealing of direct repeats from the light to the heavy strand of the mtDNA (13). This then leads to loop formation of both the heavy and the light strand of mtDNA. The initiation of loop exclusion is thought to be mediated by reactive oxygen species (13, 14).

Reactive oxygen species can damage mtDNA (15–17), and damage by hydrogen peroxide is more extensive in mtDNA than in nuclear DNA (18). Furthermore it has been shown recently that increased oxidative stress is correlated to an altered mitochondrial function in vivo (19). In addition, oxidative stress induced by solar radiation may also be responsible for the increased frequency of mtDNA mutations in photoaged human skin (14, 20–22). Evidence for a direct link between reactive oxygen species and the generation of large deletions of the mitochondrial genome, however, has not yet been provided.

In the present study we demonstrate that the common deletion can be generated under defined conditions in human dermal fibroblasts through repetitive UVA irradiation. By employing this in vitro model we provide evidence that UVA radiation-induced mtDNA deletions are caused by the generation of singlet oxygen.

EXPERIMENTAL PROCEDURES

Cell Culture—Normal human skin fibroblasts were cultured in Eagle’s minimum essential medium (Life Technologies GmbH, Eggenstein, Germany) containing 15% fetal calf serum (Greiner, Frickenhausen, Germany), 0.1% l-glutamine, 2.5% NaHCO3, and 1% streptomycin/ampicillin B in a humidified atmosphere containing 5% CO2. Cells were kept in 10-cm culture dishes for culture and irradiation.

Generation of the Common Deletion by UVA Irradiation—For UVA irradiation, medium was replaced by PBS, lids removed, and cells were exposed to radiation from a UVASUN 5000 Biomed irradiation device (Mutzhas, Munich, Germany). The emission was filtered with UVACRYL (Mutzhas) and UG1 (Schott Glasswerke, Munich, Germany) and consisted of wavelengths greater than 340 nm. The UVA output was determined with a UVAMETER (Mutzhas) and found to be approximately 70 milliwatts/cm2 UVA at a tube-to-target distance of 30 cm.

In order to generate the common deletion, cells were irradiated three times daily with 8 J/cm2 UVA for 4 consecutive days and checked for viability by trypan blue exclusion. Cells were then aliquoted 1:1 with one aliquot stored at −80 °C until extraction of mtDNA. The other aliquot was plated to a 10-cm culture dish for ongoing culture and irradiation as indicated.

Chemical Treatments and Singlet Oxygen Generation—All chemicals were purchased from Sigma except for sodium azide (Merck) and applied as described previously (23). Vitamin E (as α-tocopheryl succinate) was dissolved in ethanol and added to cells 24 h before irradiation at a concentration of 25 μM. For irradiation in the presence of heavy water, deuterium oxide (99.9 atom % 2H) was used at a final concentration of 95% in PBS (24). Singlet oxygen was generated by thermal decomposition of the endoperoxide of the disodium salt of 3,3'-1,4-naphthylidene}
In Vitro Generation of the Common Deletion by UVA

**Scheme 1. Model for generation of common deletion via UVA-induced singlet oxygen (modified from Shoffner et al. (9)).**

A, replication of mtDNA commences at the origin of the heavy strand (O_H) separating the light from the heavy strand. Abbreviations used are: DR1, direct repeat 1 (empty box); DR2, direct repeat 2 (solid box); OL, origin of light strand replication. B, DR1 of the heavy strand and DR2 of the light strand misanneal generating a downstream loop of the heavy strand. Due to a high content of guanines it is particularly susceptible to strand breaks generated by UVA irradiation or other sources of singlet oxygen. C, after degradation of the excluded loop and ligation of the free ends of the heavy strand, replication can be completed, leading to a normal (left) and a deleted (right) mtDNA molecule.

diene/dipropionate (NDPO2). Cells were incubated with 0.3 mM NDPO2 in PBS for 1 h in the dark at 37 °C, which yielded singlet molecular oxygen and 3,3′,4,4′-tetranitrophenylethylene/dipropionate (NDP) (25).

This singlet oxygen system was shown to be well suited for use in cell cultures, because it is water-soluble and nontoxic for these cells up to 40 mM for 1-h incubation (24). Infrared emission of singlet oxygen was measured with a liquid nitrogen-cooled germanium photodiode detector (model EO-817L, North Coast Scientific, Santa Rosa, CA) as described previously (25). The rate of singlet oxygen generation was monitored by diagnostic digestion with the restriction enzyme Hae III (New England Biolabs GmbH, Schwalbach, Germany). Cells were incubated with 0.3 mM NDPO2 in PBS for 1 h in the dark at 37 °C, which yielded singlet molecular oxygen and 3,3′,4,4′-tetranitrophenylethylene/dipropionate (NDP) (25).

**DNA Extraction**—Total cellular DNA was extracted from normal human fibroblasts employing the QIAamp Tissue Kit (Qiagen, Hilden, Germany).

**PCR Analysis**—For estimation of mtDNA content, PCR with primers C1 and C2 was carried out, amplifying a product of 247 base pairs in length. Amplification of fragments representing the common deletion was carried out as described previously (20, 26). In brief, primer oligonucleotides (A1/A2) were designed to anneal outside the common deletion, allowing the efficient amplification of the shorter and deleted mtDNA-fragments. To increase sensitivity and specificity a secondary, nested PCR was performed (B1/B2) from the primary PCR product.

Linear amplification conditions for each primer pair were determined as described in detail previously (27). Amplification was found to be linear up to 31 cycles for primers C1/C2 and A1/A2 and up to 30 cycles for B1/B2. Therefore primary PCR (primers A1/A2 and C1/C2) was carried out in 100-μl reaction volume with 0.1–0.3 μg of genomic DNA and 0.5 unit of Taq polymerase. Primer and nucleotide concentrations were 1 and 400 μM, respectively. For PCR, initial denaturation (94 °C, 4 min) was followed by 28 cycles of denaturation (94 °C, 1 min), annealing (58 °C, 1 min), and extension (72 °C, 45 s). A 2-μl aliquot of the primary reaction was entered into reamplification (B1/B2) with the same PCR conditions except concentration of dNTPs (40 μM/dNTP), an annealing temperature of 68 °C, and a further decrease in the extension time to 30 s. Polymerase chain reaction was carried out in a Perkin-Elmer DNA Thermocycler 480 (Perkin-Elmer Applied Biosystems, Weiterstadt, Germany), primer oligonucleotides were generated by MWG-Biotech (Ebersberg, Germany).

**Quantification by Ion-Exchange Chromatography**—Quantitation was performed by ion-exchange chromatography connected to an on-line ultraviolet spectrophotometer (Gynkotek, Germering, Germany), which allowed exact quantification of amplification products at 260 nm (27, 29), and quantitation of deleted mtDNA was carried out as described previously (20). The amplification product generated by primer pair C1 and C2 represents a segment of the mtDNA (30), carrying no known mutations of the mtDNA. Thus, it served as a reference fragment representing the overall amount of mtDNA molecules present in the cultured cells. Values determined for PCR products representing the common deletion were normalized to values of the reference fragments of the same cell lines.

**Restriction Enzyme Analysis**—To confirm their identity, PCR products were subjected to diagnostic digestion with the restriction enzyme XbaI (New England Biolabs GmbH, Schwalbach, Germany).

**RESULTS**

Generation of the Common Deletion in Normal Human Fibroblasts by Repetitive UVA Irradiation—In order to determine the maximal UVA dose that allows sublethal repetitive irradiation, normal human fibroblasts were exposed to UVA doses of 0, 4, 8, and 16 J/cm² three times daily (Fig. 1). Repetitive irradiation with 16 J/cm² induced cell death, whereas irradiation with doses of 0, 4, and 8 J/cm² had no effect on cell viability.
viability. In the subsequent experiments cells were therefore exposed to repetitive doses of 8 J/cm².

Amplification of total DNA extracts from normal human fibroblasts with primer oligonucleotides C1/C2 by PCR yielded the expected fragment of 247 base pairs in all experiments. The amplification of this fragment confirmed the effective extraction of mtDNA and served as a reference fragment for subsequent quantification (Fig. 2a). Nested PCR of fibroblasts exposed to repetitive UVA irradiances followed by ion-exchange chromatography showed no signal for the common deletion after 12 irradiations. Exposure of cells to 24 irradiations yielded the first detectable signal (Fig. 2b and c), and maximal induction was observed after an irradiation of cells for 36 times. Sham-irradiated control cells showed no increase of the common deletion at any of the irradiation intervals. Digestion of PCR products with the restriction enzyme XbaI confirmed the identity of the amplified fragments (data not shown).

Role of Singlet Oxygen in the Generation of the Common Deletion—The role of singlet oxygen as a mediator of UVA radiation effects has been shown for several systems (31, 32). Therefore, in the present study, reagents capable of quenching (sodium azide, vitamin E) or enhancing (deuterium oxide) singlet oxygen effects were examined whether these UVA radiation-induced effects could be mimicked by stimulating unirradiated cells with singlet oxygen generated via thermal decomposition of NDPO2 (23, 25). As shown in Fig. 3, incubation of cells with NDPO2 led to the induction of the common deletion in normal human fibroblasts parallel to the observed UVA induced mutagenesis, whereas incubation with NDP did not generate a signal for the common deletion.

DISCUSSION

Generation of the Common Deletion in Vitro with Physiological Doses of UVA—Large scale deletions of mitochondrial DNA such as the common deletion are thought to play a central role in aging of human tissues. Analysis of the mechanism by which the common deletion is generated in human cells during the normal aging process would require an assay system allowing the generation of mitochondrial mutations under standardized conditions and in a reproducible manner by exposing relevant target cells to physiologically occurring oxidative stress. In the present study we have therefore assessed whether it is possible to generate mitochondrial DNA mutations in cultured human dermal fibroblasts by exposing them in vitro to UVA radiation.

The assay system is based on the following. Chronically sun-exposed human skin has a higher content of mtDNA mutations than sun-protected skin, and a role for mtDNA mutations in photoaging of human skin has been proposed (14, 20, 22). Comparative analysis of the epidermal versus dermal compartment of photoaged skin revealed that the common deletion
Singlet oxygen generation was monitored by the formation of the dark at 37 °C, and yielded singlet molecular oxygen and NDP. The irradiated cells with D₂O: for UVA irradiation of fibroblasts, in the present study, dermal fibroblasts were used and exposed to UVA radiation, which can penetrate into the dermis at significant dose levels. The UVA radiation doses employed for in vitro irradiation of fibroblasts are of physiological relevance, since they are similar to those administered to human skin during the course of a 15–30-min sun exposure on a summer day at noon at the northern latitude of 30–35° (33). Human cells are capable of repairing damage to mtDNA induced by reactive oxygen species (34, 35), and therefore an irradiation regimen has been developed that shifts the steady-state in cells between irradiation-induced damage and ongoing repair toward the damage side. By employing this protocol, it has indeed been possible to reproducibly generate the common deletion in human fibroblasts under standard conditions (Fig. 2).

A possible explanation for this could be that cells harboring the common deletion show a selective growth advantage, thus leading to increasing amounts of this deletion. It has been described, however, that there are selection mechanisms in cells containing large scale mtDNA deletions that lead to a growth disadvantage rather than an a growth advantage (26, 36). Therefore it is very unlikely that the observed increase of the common deletion is simply due to differences in growth. Furthermore, the induction of the common deletion was a function of the number of UVA radiation exposures given to cells, indicating that it resulted from cumulative photodamage. We therefore propose that UVA radiation-induced generation of the common deletion in human dermal fibroblasts, as described here, represents a novel in vitro model to study the mechanism (i) by which the common deletion is generated in human cells in general and (ii) by which UVA radiation contributes to aging in particular.

**Single Strand Breaks by Singlet Oxygen**—It has been proposed that oxidative stress is responsible for the generation of mitochondrial DNA mutations in human cells (5), but a direct link between reactive oxygen species and large scale deletions of mtDNA has thus far been elusive. Here we have demonstrated that UVA radiation causes the generation of the common deletion in human fibroblasts through an oxidative mechanism that depends on the generation of singlet oxygen. Evidence for the involvement of singlet oxygen in biological processes is based on the use of quenchers to diminish singlet oxygen-mediated effects, on strategies that allow extension of the half-life of singlet oxygen to enhance singlet oxygen-mediated effects, and on the use of singlet oxygen-generating systems. By employing these three different strategies we here provide evidence that generation of the common deletion in human dermal fibroblasts through repetitive UVA irradiation critically depends on the generation of singlet oxygen. Formation of mitochondrial DNA mutations is thought to occur by misannealing of direct repeats situated in the D-loop (Scheme 1) during replication of mtDNA, thereby leading to excluded loops (9). In order to complete the deletion process, single strand breaks need to be generated to permit exonucleolytic degradation of the looped-out DNA. Based on the present study we propose a model in which singlet oxygen generation is responsible for base damage and subsequent strand break formation. In DNA, guanosines represent the prime targets for modification by UVA, and singlet oxygen (37, 38) and G stretches are extremely susceptible to modification (39–41). In mtDNA, replication is started at the replication origin of the heavy strand (OH) generating the D-loop (Scheme 1), which after misannealing leads to the excluded loop (9). The heavy strand contains several guanosine stretches of 3 to 5 bases of which many are in direct proximity to the direct repeats. Therefore it is tempting to speculate that the required strand breaks are indeed generated in this excluded loop via singlet oxygen.

**Relevance for Photoaging**—Our studies identify a previously unrecognized biological function of singlet oxygen. In addition, they demonstrate that oxidative stress is indeed responsible for the generation of large scale deletions of mitochondrial DNA in human cells that have been exposed to UVA radiation, which induces tissue aging under normally occurring conditions. In previous studies, singlet oxygen was found to mediate other UVA radiation-induced biological effects as well. In particular, UVA radiation-induced expression of metalloproteinases I, II, and III expression in human dermal fibroblasts was mediated through the generation of singlet oxygen (24). The increased expression of matrix metalloproteinases in human skin fibroblasts is thought to be partially responsible for the decreased content of collagen fibers in photoaged human skin. Furthermore, recent findings indicate that singlet oxygen is linked with the in vivo UVA action spectrum, which is responsible for photoaging of mouse skin (42). Taken together with the present observation that UVA radiation-induced singlet oxygen is capable of generating mitochondrial DNA mutations in UVA-irradiated dermal fibroblasts, it is possible that the generation of singlet oxygen in human skin is of central importance for photoaging. Singlet oxygen quenching may thus represent an effective strategy to protect human skin from photoaging.

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