Mitochondrial DNA Deletions in Human Skin Reflect Photo- Rather Than Chronologic Aging

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We have examined the use of mitochondrial DNA (mtDNA) as a molecular marker to study the relation between chronologic aging and photoageing in human skin. Using a 3-primer quantitative polymerase chain reaction method we have studied changes in the ratio of the 4977 bp deleted to wild-type mtDNA in relation to sun exposure and chronologic age of human skin. Based on previous studies, samples showing greater than 1% deleted mtDNA were classed as abnormal. There was a significant increase in the incidence of high levels (i.e., >1%) of the 4977 bp deleted mtDNA in sun-exposed sites (27%, 27 of 100) compared with sun-protected sites (1.1%, one of 90) (Fisher's exact test, $p < 0.0001$). There appeared to be no relation between the frequency of the mtDNA deletion and age. Analysis of split skin samples showed that most deletions (93%, n = 27) were confined to the dermal rather than the epidermal component, and in keeping with this deletions were found in three of six primary cultures of fibroblasts from sun-exposed sites. Deletions were not seen in the epidermal component of several epidermal tumors nor were deletions seen in fibroblasts cultured from an individual with Werner's syndrome. We propose that deletions or mutations of mitochondrial DNA may be useful as a marker of cumulative ultraviolet radiation exposure. ***Key words: mtDNA common deletion/nonmelanoma skin cancer/photoageing/ultraviolet radiation. J Invest Dermatol 110:149–152, 1998***

Many of the age-associated changes in skin, including the increased incidence of skin cancer, reflect the long-term effects of ultraviolet radiation rather than chronologic aging (Gilchrest and Yaar, 1992; Rees, 1997). There is a need to develop molecular markers by which to characterize this process and understand the relation between photo- and chronologic aging. We have examined mitochondrial DNA (mtDNA) as a candidate for such a marker for several reasons. Firstly, accumulation of mtDNA deletions has recently been proposed as an important contributor to aging in various cell types (Cortopassi *et al*, 1992; Wallace, 1992; Lee *et al*, 1994; Yang *et al*, 1994), the most common of the reported mtDNA deletions is the 4977 bp or "common" deletion that was originally observed in two mitochondrial myopathies, the Kearns–Sayres syndrome, and chronic progressive external opthalmoplegia (Wallace, 1992). Secondly, one recent report (Pang *et al*, 1994) has shown differences in the incidence of the 4977 bp deleted mtDNA between several skin tumors (actinic keratosis and squamous cell carcinoma) and normal skin from a single patient study. Thirdly, within a single mitochondrion different mitochondrial genomes (2–10 copies per organelle) can complement each other, which means that cells can accumulate and tolerate high (up to 90%; Chomyn *et al*, 1992; Moraes *et al*, 1993; Moraes *et al*, 1995) levels of mutated/deleted mtDNA. As a result, cells harboring defective mtDNA will be relatively free of the negative selection pressure that may affect cells harboring nuclear DNA mutations in essential genes. We have therefore used a 3 primer quantitative polymerase chain reaction (PCR) method to study changes in the ratio of the 4977 bp deleted to wild-type mtDNA in relation to sun exposure and chronologic aging in human skin.

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Abbreviation: mtDNA, mitochondrial DNA.

MATERIALS AND METHODS

Clinical samples

Sun-protected skin Skin was taken from both (i) young patients following a pinnaplasti or circumcision (n = 16; age range, 2–39 y; mean age, 10 y) and (ii) postmortem samples (buttock, inner forearm, and heel) (n = 18; age range, 41–84 y; mean age, 72 y).

Sun-exposed skin Samples were taken from (clinically normal) perilesional skin from patients attending for skin cancer excision. Thirty-five samples were prospectively classed as coming from sites of high exposure (face and hands), and 15 were from low or intermittent sun-exposed sites (i.e., trunk and leg). For statistical analysis, patients with cosmetic procedures (n = 6), who tended to be younger, were excluded so that the mean ages of the "high" and "low" sun-exposed sites were comparable (i.e., 70.8 and 64.6 y, respectively).

Epidermal neoplasms Samples were taken from a range of continually sun-exposed and intermittently sun-exposed body sites as follows: upper leg (n = 2), lower leg (n = 3), and forehead (n = 1) for squamous cell carcinomas; upper and lower leg and toe (all n = 1) for psoralen plus ultraviolet A keratoses; forehead (n = 3), ear (n = 2), hand (n = 1), and leg (n = 3) for actinic keratoses; ear (n = 2) for viral warts.

Epidermis and dermis were separated using 0.25% dispase at 4°C overnight, and DNA extracted as described (Jackson *et al*, 1992). Fibroblasts were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Paisley, U.K.) with 10% fetal bovine serum (at 37°C with 5% CO_2), and DNA extracted as described above. Fibroblasts (upper arm) from a 35-y-old patient with Werner's syndrome were a kind gift of Professor J.A.A. Hunter and Dr. G.C. Priestly (University of Edinburgh, Edinburgh, U.K.).

Three primer PCR To simultaneously detect and quantitate the ratios of both deleted and wild-type mtDNA in the skin DNA samples, we used a

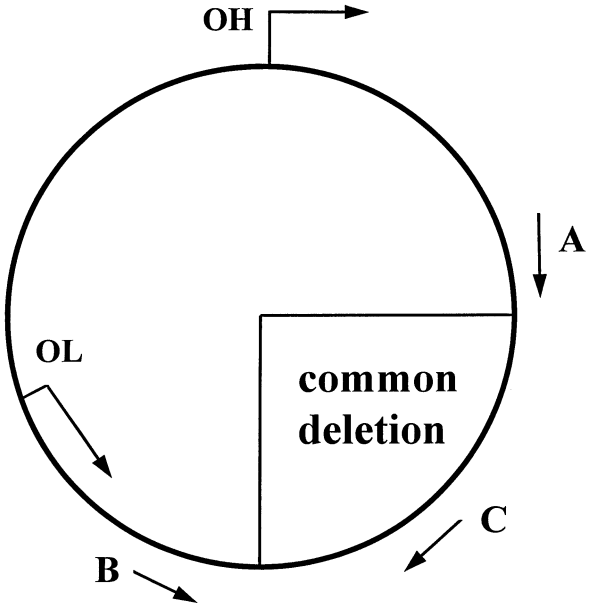

Figure 1. Schematic representation of the 3-primer PCR method. Schematic representation of the human mtDNA showing the origin of light (OL) and heavy (OH) strand replication and the approximate location of the 4977 bp common deletion. Oligonucleotide primers A, B, and C were used to simultaneously PCR amplify DNA fragments originating from both deleted and wild-type mtDNA. The PCR products originating from wild-type mtDNA (755 bp) or from deleted mtDNA (470 bp) were separated on a nondenaturing polyacrylamide gel and analyzed (see *Materials and Methods*).

3 primer PCR procedure (Sciacco *et al*, 1994) as illustrated in **Fig 1**. Primers A and C correspond to heavy strand positions 13720–13705 and 9028–9008, respectively (Anderson *et al*, 1981); primer B corresponds to light strand positions 8273–8289. Primer C maps to a mtDNA region within the common deletion, whereas primers A and B flank the deleted region. Therefore primers B and C only amplify wild-type mtDNA and primers A and B only amplify deleted mtDNA (the distance between the two primers in the absence of the deletion, ≈5.5 kb, is too long to be amplified under our PCR conditions as described below). Using three primers allowed the simultaneous detection of two bands, the larger one (755 bp) corresponding to the wild-type mtDNA, and the smaller one (470 bp) corresponding to deleted mtDNA harboring the "common deletion." The PCR reaction mixture (25 μl total volume) contained 100 ng total cellular DNA, 200 μM dNTP, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM Mg Cl_2, 0.1% Triton X-100, 2.5 U *Taq* DNA polymerase (BioTaq, Bioline UK, London, U.K.), 25 pmoles of primers A and B, 6.25 pmoles of primer C, and 3 μCi of [α-^{32} P]-dATP. The PCR conditions were 25 cycles of 94°C at 1 min, 55°C at 1 min, 72°C at 2 min including a final extension of 15 min at 72°C. These PCR products were then electrophoresed through a 6% nondenaturing polyacrylamide gel and the radioactive PCR fragments were quantitated by phophorimage analysis using the ImageQuant software (Molecular Dynamics, Chesham, U.K.). Levels of deleted mtDNA greater than 1% were considered high.

Identification of the common mtDNA deletion-junction by cloning and sequencing The 470 bp PCR product, corresponding to the deleted mtDNA harboring the "common deletion," was cloned into the pCR2.1 vector and transformed into competent cells (TA cloning kit, Invitrogen, Leek, The Netherlands) according to manufacturer's instructions. Bacterial colonies containing recombinant vector were identified and plasmid DNA was extracted by the alkaline lysis method (Maniatis *et al*, 1989). Using a sequencing primer in close proximity to the deletion junction (i.e., heavy-strand positions 13751–13550; Anderson *et al*, 1981) the DNA was sequenced using the *Sequitherm*™ cycle sequencing kit (Epicenter Technologies, Madison, WI).

Southern analysis Two micrograms of total DNA extracted from both the epidermis and the dermis of perilesional skin biopsies were digested with *Pvu* II, electrophoresed through a 0.6% agarose gel, and transferred to Hybond-N membrane (Amersham International, Buckinghamshire, U.K.). The membrane was hybridized with a ^{32}P-labeled probe corresponding to the mtDNA D-Loop region (positions 15977–16420; Anderson *et al*, 1981) according to the manufacturer's instructions. The ratio of deleted mtDNA:total wild-type DNA was determined by phosphorimage analysis using the Imagequant software (Molecular Dynamics).

Table I. The incidence of the 4977 bp deleted mtDNA in sun-exposed and sun-protected skin

% level 4977 bp deleted mtDNA	Pinnaplasti and circumcision, number of samples[a]	Post-mortem, number of samples[a]	Perilesional, number of samples[a]
<1[b]	32 (100%)	89 (98.9%)	73 (73%)
1–5	0	1 (1.1%)	11 (11%)
5–10	0	0	7 (7%)
10–20	0	0	5 (5%)
>20	0	0	4 (4%)
Total >1[c]	0	1 (1.1%)	27 (27%)

[a]Total number of individual DNA samples extracted from both the epidermis and the dermis from different body sites of the patients (see *Materials and Methods* for details). Sun-protected skin: n = 32 (pinnaplasti or circumcision), n = 90 (postmortem). Sun-exposed skin: n = 100 (perilesional skin).
[b]Levels of deleted mtDNA <1% were considered background (see text)
[c]Levels of deleted mtDNA >1% were classed as high (see text).

RESULTS

The 3 primer quantitative PCR assay detects the 4977 bp deleted mtDNA and reflects the amount of the deletion The 470 bp PCR bands from six patients were cloned and sequenced to confirm the specificity of the PCR primers and to verify that the 470 bp PCR product contained the characterstic deletion-junction created by the common deletion. This deletion-junction sequence (i.e., 5′-TAC-CACCT acctccctcacca$^{8482bp\backslash 13460bp}$TTGGCAG-3′) was identified exclusively in each of the cloned PCR bands. Characteristically this sequence contained only one of the two 13 bp repeats that flank the common deletion in wild-type mtDNA (lower case letters); furthermore, the 5′-side of the deletion junction contained the ATPase 8 gene sequence whereas the immediate 3′-side contained sequences of the ND5 gene that are normally 5 kb in the 3′ direction. This junction is identical to that observed in patients with the common deletion in mitochondrial myopathies (Schon *et al*, 1989).

Results comparable with the 3-primer PCR assay were obtained by Southern analysis (data not shown), confirming that the PCR assay reflects the amount of deleted mtDNA in the skin samples.

mtDNA deletions are more common in skin from sun-exposed sites than sun-protected sites and there is no evidence of association with age As shown in **Table I**, almost all of the sun-protected epidermal and dermal samples [32 of 32 (100%) pinnaplasti/circumcision and 89 of 90 (99.%) postmortem samples] showed either no deletion or only a background level (i.e., <1%) of the 4977 bp deleted mtDNA. By contrast, high levels (i.e., >1%) of deleted mtDNA were found in 27 of 100 (27%) epidermal or dermal samples from perilesional sites ("sun exposed") (Fisher's exact test, $p < 0.0001$). Sixteen of these 27 samples showed levels of deleted mtDNA greater than 5%. All 27 samples showing high levels of deleted mtDNA were from areas of high sun exposure (face and hands) and none were from areas of intermittent sun exposure (trunk and leg). In one individual two biopsies, one from the face and one from the trunk, were available; dermis from the face showed a level of deleted mtDNA of 3.7%, whereas no significant deleted mtDNA (i.e., <1%) was found in the trunk sample.

The increased frequency of deleted mtDNA in sun-exposed rather than sun-protected skin is unlikely to be explained by age differences, as the mean ages of the high sun-exposed patients and the sun-protected groups were comparable (high sun-exposed *versus* intermittant sun-exposed perilesional samples, mean ages 71 *vs* 65 y, $p = 0.15$; high sun-exposed perilesional samples *versus* sun-protected postmortem, mean ages 71 *vs* 72 y, $p = 0.77$). Furthermore there was no significant correlation with patient age for the 27 samples showing high levels of deleted mtDNA ($p = 0.39$, rho = 0.18, Spearman's rank correlation).

High levels of 4977 bp deleted DNA occur almost exclusively in the dermis rather than in the epidermis (Figure 2) Twenty-

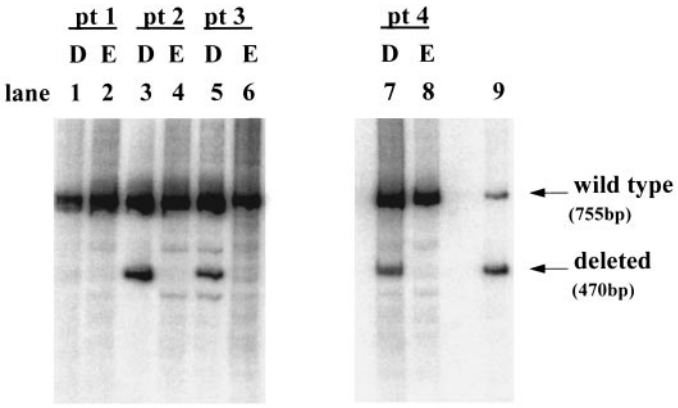

Figure 2. Phosphorimage showing the increased frequency of 4977 bp deleted mtDNA in dermis compared with the epidermis of sun-exposed skin. The 3-primer PCR method was used to amplify DNA fragments originating from both deleted and wild-type mtDNA in the dermis and epidermis of sun-exposed skin. *Lanes 1–8* represent DNA amplified from four sun-exposed perilesional skin samples, i.e., Pt1–4 (D, dermis; E, epidermis); *lane 9* is amplified mtDNA from the muscle of a patient with a known mitochondrial disorder and is consequently harboring a heteroplasmic mtDNA deletion (≈75% deleted and 25% wild-type mtDNA), this lane therefore serves as an internal control for the experiment. The phosphorimage clearly shows that for the same patient the dermis shows a greatly increased level of deleted mtDNA compared with the epidermis.

five of the 27 samples (93%) that harbored a high level of deleted mtDNA were dermal, with only two epidermal samples showing high levels of mtDNA deletions. In these two epidermal samples, however, the corresponding dermis showed significantly higher levels of deletion (5- and 10-fold), suggesting that contamination of epidermis with dermis may have occurred during preparation of the split skin samples. Because fibroblasts are the main cell type of the dermis, we examined skin fibroblast DNA and found deleted mtDNA in three of six primary cultures of fibroblasts (mean age 72 y) from sun-exposed sites, which confirms that fibroblasts can harbor high levels of deleted mtDNA. We could not detect any deleted mtDNA from skin fibroblasts of a patient with Werner's syndrome (Gawkrodger *et al*, 1995).

No evidence of mtDNA deletions in a range of skin neoplasms Because of the possibility of deleted mtDNA resulting from dermal contamination, paraffin-embedded epidermal neoplasms (actinic keratoses, n = 9; psoralen plus ultraviolet A keratoses, n = 3; viral warts, n = 2; and squamous cell carcinomas, n = 6) were microdissected. Samples were taken from a range of continually sun-exposed and intermittently sun-exposed body sites (see *Materials and Methods*). No sample showed >1% deleted mtDNA.

DISCUSSION

mtDNA deletions reflect photo- rather than chronologic aging Previous studies in several human tissues (Cortopassi *et al*, 1992; Lee *et al*, 1994; Barreau *et al*, 1996; Fahn *et al*, 1996) have reported a background frequency of <1% for the 4977 bp deleted mtDNA and so in our study samples showing a frequency greater than 1% were classed as harboring "high" levels of 4977 bp deleted mtDNA. These high levels of deleted mtDNA are unlikely to be artifacts of the 3 primer PCR assay for several reasons. Firstly, we have shown that the 470 bp PCR band corresponds only to DNA harboring the characteristic 4977 bp common deletion. Secondly, the 3 primer quantitative PCR assay reflects the amount of deleted mtDNA as determined by Southern analysis (present study and Sciacco *et al*, 1994). Thirdly, high levels of 4977 bp deleted mtDNA have been previously observed in one preliminary study of skin, where a value of 12% was reported (Pang *et al*, 1994). In addition there have been several reports of high levels in mitochondrial myopathies of various tissues where values can be as high as 95% of total mtDNA (McShane *et al*, 1991; Wallace, 1992; Moraes *et al*, 1995; Collombet *et al*, 1996).

Our results show that almost all the sun-protected epidermal and dermal samples as well as the epidermal neoplasms showed only a background level (<1%) of mtDNA deletions that is in agreement with reported levels of deleted mtDNA in other tissues (Cortopassi *et al*, 1992; Lee *et al*, 1994; Barreau *et al*, 1996; Fahn *et al*, 1996). By contrast there was a significant increase in the frequency of high levels (>1%) of deleted mtDNA in sun-exposed compared with sun-protected sites. Furthermore, all 27 samples showing high levels of deleted mtDNA were from areas of chronic sun exposure (face and hands) and none were from areas of intermittent sun exposure (trunk and leg). These observations suggest an association between the increased frequency of high levels of deleted mtDNA and chronic sun exposure. Although it has been suggested that the accumulation of mtDNA deletions is an important contributor to the aging process (Cortopassi *et al*, 1992; Wallace, 1992; Lee *et al*, 1994; Yang *et al*, 1994), in this study there was no significant relation between the presence of >1% deleted mtDNA and chronologic age.

Putative mechanism Any mechanism explaining our observations must address both the differences between epidermis and dermis, and ultraviolet radiation as the major agent involved in photoageing. Based on previous studies in several tissues (Ikebe *et al*, 1990; Corral-Debrinski *et al*, 1991; Yen *et al*, 1991; Simonetti *et al*, 1992; Yang *et al*, 1994) it has been proposed (Cortopassi *et al*, 1992; Lee *et al*, 1994) that there is more 4977 bp deleted mtDNA in tissues that turn over slowly or not at all (e.g., brain and muscle) than in those that turn over relatively more rapidly (e.g., liver and blood cells). According to this proposal the high turnover of keratinocytes would result in little time to accumulate mtDNA deletions, whereas by contrast in fibroblasts the low proliferative rate would result in an accumulation of deleted mtDNA. The situation with respect to keratinocyte stem cells is unclear and worthy of study.

As to the exact mechanism leading to the 4977 bp deletion, it has been suggested that an intragenomic recombination event via slipped strand mispairing may occur at the 13 bp repeats that flank the common deletion (Schon *et al*, 1989; Shoffner *et al*, 1989; Mita *et al*, 1990; Degoul *et al*, 1991). This mechanism, however, requires regions of single-stranded DNA on both the heavy and the light mtDNA strands, but neither of the 13 bp repeats are simultaneously single-stranded during replication (Schon *et al*, 1989). It has been suggested (Schon *et al*, 1989) that homopyrimidine stretches in the 13 bp repeat and/or flanking AT-rich regions may be susceptible to DNA bending that would allow a small region or "bubble" of single-stranded DNA to open. Interestingly, these DNA stretches map to structurally labile "hot-regions" for the 4977 bp common deletion that assume an unusual bent-DNA structure and that may therefore enhance the intragenomic recombination event (Hou and Wei, 1996). Although we have no clear mechanism to explain our results, we propose that prolonged ultraviolet radiation exposure could either directly (by inducing base substitutions as opposed to deletions) or indirectly (by induction of free radicals) affect these structurally labile sites through opening a "bubble" of single-stranded DNA that would enhance the recombination event, thereby eliciting an increase in mtDNA deletions.

In conclusion, we report that changes in the incidence of the 4977 bp deleted mtDNA reflect photo- rather than chronologic aging. We have investigated the 4977 bp deleted DNA not only because it is the most common deletion in other tissues but also because it's frequency can be easily measured by an established quantitative PCR assay. The use of the 4977 bp common deletion as an index of overall damage to skin mtDNA may be relatively insensitive, however, and future work should focus on other deletions or mutations of mtDNA that may be considerably more common.

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