Induction of the Photoaging-Associated Mitochondrial Common Deletion *In Vivo* in Normal Human Skin

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Mutations of mitochondrial (mt) DNA such as the 4977 base-pair large-scale deletion, also called common deletion, are increased in photoaged skin. Direct evidence for their induction by chronic exposure to ultraviolet (UV) radiation *in vivo* in human skin has remained elusive however. Furthermore, their fate after induction is unclear. Previously unirradiated skin of 52 normal human individuals was repetitively exposed to physiological doses of UVA light. Skin and blood specimens were investigated for the presence of mtDNA mutations employing semiquantitative nested PCR, as well as real-time PCR, after 2 weeks of UV exposure and the content of the common deletion was followed up for up to 16 mo after cessation of irradiation. As assessed by both methods, repetitive UV exposure led to an approximately 40% increase in the levels of the common deletion in normal human skin. The majority of deletions were detectable in the dermis also showing the biggest increase, whereas in the epidermis only residual levels and no increase were found. Nine individuals were examined up to 16 mo after cessation of UV exposure and some showed accumulation up to 32-fold. Thus, mtDNA mutations are induced in the human skin by repetitive UV exposure. In addition, these mutations seem to represent long-term *in-vivo* biomarkers for actinic damage in the human skin.

Key words: biomarker/mitochondria/mitochondrial DNA/ultraviolet light
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Photoaged skin is characterized by wrinkles, uneven pigmentation, brown spots, laxity, and a leathery appearance. These changes are caused by chronic exposure of the skin to ultraviolet (UV) light (Berneburg *et al*, 2000; Hadshiew *et al*, 2000; Griffith, 2001). In recent years, lifestyle changes have led to an increasing exposure of people in industrialized nations to ambient and artificial UV radiation (Ananthaswamy *et al*, 1997). Current mechanistic pathophysiological concepts of photoaging comprise the UV-induced activation of transcription factors AP-1 and NFkB and the resulting expression of matrix metalloproteinases in the human skin (Fisher *et al*, 1996; Fisher *et al*, 1997; Scharffetter-Kochanek, 1997; Scharffetter-Kochanek *et al*, 2000). Furthermore, photoaged skin is characterized by increased mutations of the mitochondrial (mt) genome (Yang *et al*, 1994; Yang *et al*, 1995; Berneburg *et al*, 1997). Intraindividual comparison studies have revealed that e.g., a 4977 base pair deletion of mtDNA, the so-called common deletion, is increased up to 10-fold in photoaged skin, as compared with sun-protected skin of the same individuals (Ray *et al*, 2000). The amount of the common deletion in human skin does not correlate with chronological aging (Birch-Machin *et al*, 1998), and it has therefore been proposed that mtDNA mutations such as the common deletion represent molecular markers for photoaging (Birch-Machin *et al*, 1998). However, it is currently not known whether mtDNA mutations can be induced in human skin by chronic UV radiation exposure, and if they remain in the skin as long-term *in-vivo* biomarkers for actinic damage.

Abbreviations: bp, base pair; MED, minimal erythema dose; mtDNA, mitochondrial DNA; PCR, polymerase chain reaction; ROS, reactive oxygen species; UCA, urocanic acid; UV, ultraviolet

[1]These authors contributed equally to this work.

Results

Generation of the common deletion in human skin The common deletion could be detected in all skin specimen, indicating that unexposed as well as exposed skin sites carried the common deletion. This was the case with both PCR methods, nested PCR (Table I) and real-time PCR (Table II).

Nested-PCR analysis of irradiated skin samples showed an increase of the common deletion in 73.1% or 69.2% compared with 53.8% or 50.0% in unirradiated skin samples, respectively, depending on the defined criterion where 0% increase represents a decrease of the common deletion to zero, 50% represents no increase, and 100% increase to absolute maximum levels (see Materials and Methods, Table I, Fig 1) yielding p-values <0.01 for statistical comparisons. The quantitative magnitude of the

Table I. Baseline characteristics of study subjects

	Variable	
	N	(%)
Gender		
Male	21	(40)
Female	31	(60)
Total	52	(100)
Skin type		
I	1	(2)
II	23	(44)
III	20	(39)
IV	8	(15)
Total	52	(100)
Age		
$\leqslant 25$	24	(46)
>25	28	(54)
Total	52	(100)
Median (lower, upper quartile)	26	(21.5, 29)

induction of the common deletion was on average 41% higher after irradiation when compared with baseline ($p < 0.01$).

Real-time PCR analysis of irradiated skin samples showed an increase of the common deletion in 64.7% or 56.9% compared with 54.2% in unirradiated skin samples, respectively, depending on the defined criterion (see Materials and Methods, Table II, Fig 1). The obtained p-values were 0.040 and 0.049 for soft and strong criterion, respectively, for irradiated samples and 0.839 and 0.523, respectively, for unirradiated samples. The quantitative magnitude of the induction of the common deletion was on average 40% higher after irradiation when compared with baseline (p 0.001).

Statistical analysis of covariables (gender, age, skin type) in this cohort showed no significant influence on the effect of irradiation.

UVA-induction of the common deletion exclusively in the dermis In order to identify where in the skin the common deletion is induced, we investigated split-skin samples ($n = 5$), separated into the dermis and epidermis from UVA or non-UVA exposed skin of volunteers (Fig 2). The investigated samples showed baseline levels for the common deletion in the epidermal compartment with no increase after UVA irradiation. However, the dermis showed higher initial levels as well as a significant increase of the common deletion following repetitive UVA-exposure.

Persistence of UVA-induced common deletion for up to 16 mo In order to learn more about the fate of the common deletion after UVA-induction, nine individuals were reassessed for the level of UVA-induced mtDNA mutations after 5, 8, 10, and 16 mo.

Nested PCR Of the nine individuals, one did not yield a detectable PCR product, one showed a decreased and four showed the same levels as immediately after the irradiation regimen. However, three of the nine investigated individuals showed an increase of the common deletion over an observation period of 16 mo (Fig 3). The increase of PCR products in these individuals was 11-fold, 15-fold, and 32-fold, respectively, compared with the initially induced levels detected immediately after repetitive UVA exposure.

Real-time PCR Of the nine individuals in one sample, no material was left from the long-term time point. One individual showed a decrease and one showed the same levels as immediately after the irradiation regimen.

Six of the nine investigated individuals, however, showed an increase of the common deletion over the observation period of 16 mo (Fig 3*d*). The increase of PCR products in these individuals was 4-fold ($n = 3$), 6-fold, 16-fold, and 25-fold, respectively, compared with the initially induced levels detected immediately after repetitive UVA exposure.

Discussion

Induction of the photoaging-associated common deletion in human skin These data demonstrate the induction of a known marker mutation of mtDNA by repetitive UVA irradiation under standardized, reproducible conditions *in vivo* in human skin. This was carried out employing two separate PCR methods, nested PCR and real-time PCR. Both methods demonstrated a statistically significant increase of the common deletion in irradiated skin samples, whereas they did not reveal significant changes in unirradiated samples (Tables I, II). The lower increase of the common deletion as detected by real-time PCR, particularly

Table II. Differences of relative and dichotomized changes in the common deletion between baseline and follow-up measurements in irradiated samples when using nested PCR

	Median relative difference	Interquartile range of differences	p-Value	Increase in common deletion (% soft criterion)	p-Value	Increase in common deletion (% strong criterion)	p-Value
Irradiated samples	+0.41	1.72	<0.01	73.1	<0.01	69.2	<0.01
Unirradiated samples	+0.25	1.92	0.07	53.8	0.84	50.0	0.83

Explanation of criteria: see the section Statistical analysis; values for "Increase in common deletion" of 50% indicate no change after irradiation. A value of 100% indicates perfect correlation between irradiation and *increase* of the common deletion. A value of 0% indicates perfect correlation between irradiation and *decrease* of the common deletion. Number of studied individuals $n = 52$.

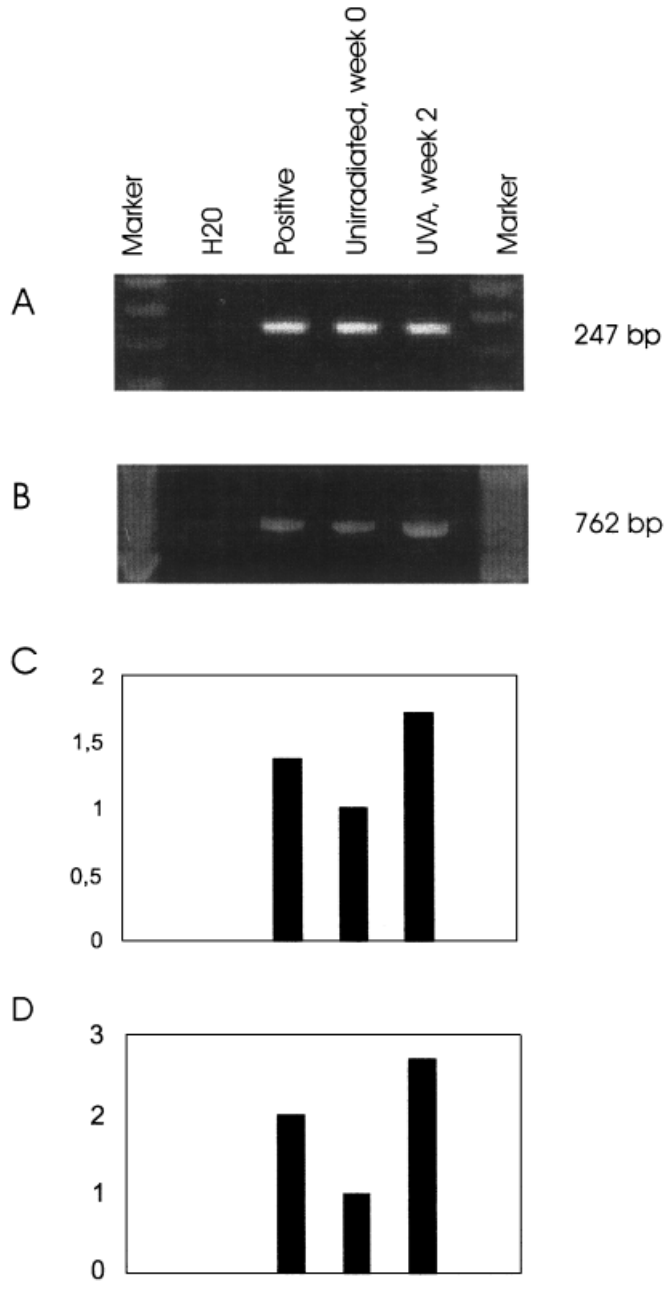

Figure 1
Induction of the common deletion *in vivo* in normal human skin and absence of a systemic effect: Normal human individuals were exposed to UVA three times daily for up to 2 wk. Of the acquired samples, after each week total cellular DNA was extracted and subjected to PCR, amplifying either the reference fragment representing the total mitochondrial genome or the fragment representing the common deletion. Positive control represents a sample of a patient with known disease caused by the common deletion. Data are plotted in arbitrary units. (*A*) Representative agarose gel of PCR amplifications of the reference fragment. (*B*) Representative agarose gel of PCR products representing the common deletion. (*C*) Quantification with Storm 860 Phosphorimager. PCR products were separated electrophoretically and stained with Vistra Green dye for 16 h. Quantification based on green fluorescence of products was subsequently carried out by phosphorimager analysis. Values determined for PCR products representing the common deletion (b) were normalized to values of the reference fragments (a) of the same skin sample. (*D*) Representative result of real-time PCR for the common deletion. Results were calculated employing the ΔΔCT method and are shown as factors of induction (For raw data, see Table IV).

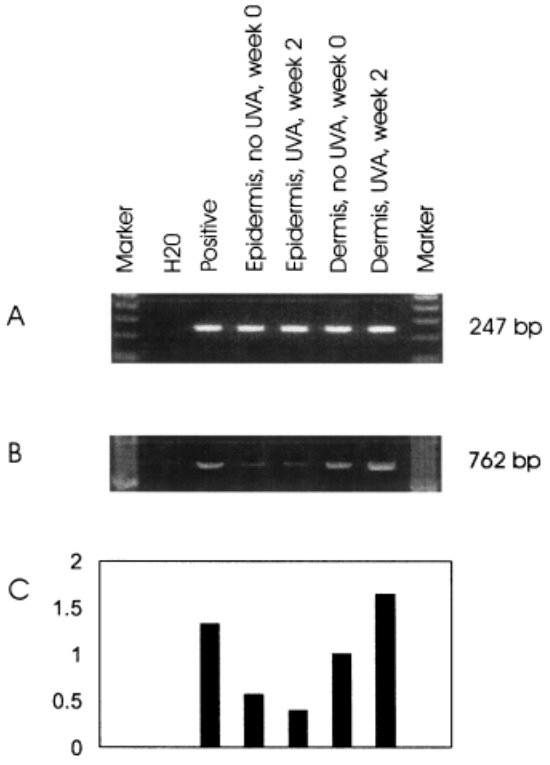

Figure 2
Amount of the common deletion in the dermis and epidermis before and after repetitive UVA-exposure: The dermis and epidermis of UVA or non-UVA exposed skin samples were separated by overnight incubation in 1 M NaCl for 48 h at 4°C. Total cellular DNA of the dermis and epidermis was extracted and subjected to PCR, amplifying either the reference fragment representing the total mitochondrial genome, or the common deletion. Positive control represents a sample of a patient with known disease caused by the common deletion. Data are plotted in arbitrary units. (*A*) Representative agarose gel of PCR amplifications of the reference fragment. (*B*) Representative agarose gel of PCR products representing the common deletion. (*C*) Quantification with Storm 860 Phosphorimager. PCR products were separated electrophoretically and stained with Vistra Green dye for 16 h. Quantification based on green fluorescence of products was subsequently carried out by phosphorimager analysis. Values determined for PCR products representing the common deletion (b) were normalized to values of the reference fragments (a) of the same skin sample.

when applying the "strong criterion", may be partially explained by the fact that when, up to 6 y after ascertainment of samples, real-time PCR was carried out, some of the samples failed to yield PCR results, thus reducing statistical power. Conversely, the mean relative difference, as assessed by the two separate PCR methods, was + 0.41 with nested and +0.40 with real-time PCR. Thus, both methods revealed almost identical median increases of the common deletion in normal human skin following repetitive UV-irradiation.

It has previously been reported that the common deletion can be induced by repetitive UV exposure in a singlet oxygen-dependent fashion in normal fibroblasts *in vitro* (Berneburg *et al*, 1999). *In vivo*, however, previous publications could thus far only report an association between chronically sun-exposed skin showing signs of photoaging and the presence of mtDNA mutations (Berneburg *et al*, 1997; Birch-Machin *et al,* 1998). In contrast, this work demonstrates a direct cause/effect relationship for ultraviolet light and the generation of mtDNA mutations in human

skin. In contrast to *in vitro* experiments, where prior to UVA-exposure, the common deletion could not be detected, *in vivo* in human skin all 52 tested individuals showed initial background levels of this mutation. This finding is in line with reports from other groups and ourselves, indicating that normal human skin does contain mtDNA mutations (Yang *et al*, 1994; Yang *et al*, 1995; Berneburg *et al*, 1997; Birch-Machin *et al*, 1998; Ray *et al*, 2000). It is not clear as to which factors determine the level of this background mtDNA mutational level, however. Only future studies may clarify whether this is due to interindividual differences in cumulative UV exposure, antioxidant capacity, or both.

UVA-induction of the common deletion exclusively in the dermis It has been reported that the common deletion can be induced not only in fibroblasts (Berneburg *et al*, 1999) but also in cultured keratinocytes by repetitive UV exposure (Koch *et al*, 2001). The common deletion, however, could not be detected in the epidermis of unirradiated volunteers (Birch-Machin *et al*, 1998). Employing highly sensitive nested PCR, we could detect the common deletion in the epidermis in our system. Levels were, however, small compared with the detected amounts in the dermis and the above-described induction by UVA occurred exclusively in the dermis. It seems likely that the mechanism of generation of the common deletion exists in all UV-exposed cells. However, the functional relevance of the common deletion in the epidermis with its high turnover of cells seems questionable. Fibroblasts usually rest at G0 of the cell cycle and are not shed in the course of skin turnover as keratinocytes are, thus rending dermal fibroblasts more vulnerable to UVA-induction of the common deletion.

The common deletion persists *in vivo* Following induction, the common deletion was detectable for up to 16 mo as assessed by nested and real-time PCR, indicating that mtDNA mutations may possibly be of a long-lasting or even permanent nature in human skin. Thus, the imprint of mutations into mtDNA may serve as a long-term biomarker for UV-damage inflicted during an individual's lifetime. However, follow-up investigations are necessary to consolidate this hypothesis for a time period longer than 16 mo to show whether in the irradiated sites this translates into actual actinic damage at later stages.

In a number of long-term follow-up samples (nested PCR 3/9, real-time PCR 6/9), the levels of the common deletion in irradiated skin continued to increase with a magnitude up to 32-fold for nested PCR and 25-fold for real-time PCR. It has been postulated for the normal aging process (Cortopassi and Arheim, 1990; Richter, 1995; Yakes and Van Houten, 1997; Wei, 1998; Wallace, 1999; Bohr, 2002; Xu and Finkel, 2002) as well as for photoaging that the induction of ROS generates mtDNA mutations, in turn leading to a defective

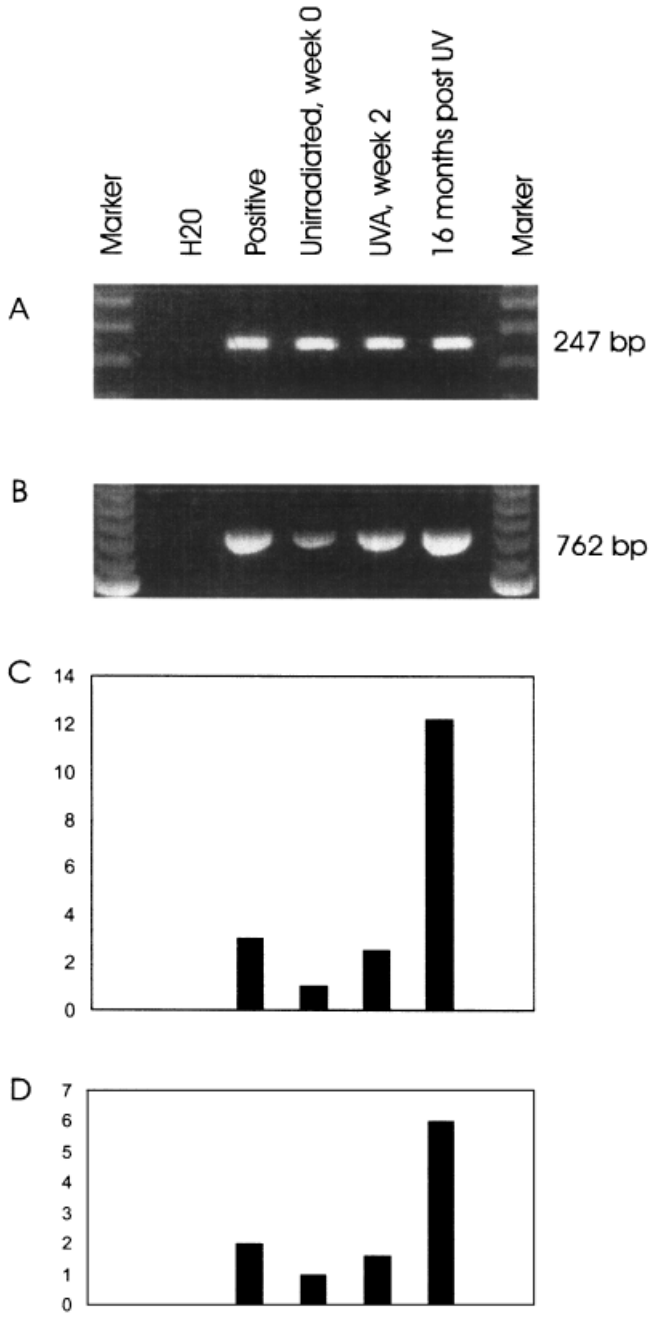

Figure 3
Persistence of UVA-induced common deletion for up to 16 mo: Normal human individuals were repetitively exposed to UVA in order to induce the common deletion. After cessation of irradiation, samples were taken after 5, 8, 10, and 16 mo. Total cellular DNA was extracted and subjected to PCR, amplifying either the reference fragment representing the total mitochondrial genome, or the common deletion. Positive control represents a sample of a patient with known disease caused by the common deletion. Data are plotted in arbitrary units depicting absolute values of PCR-fragments. Expression of data as fold induction as described in the text is calculated from two values: (i) common deletion/reference fragment of unirradiated skin versus common deletion/reference fragment of irradiated skin immediately after irradiation regimen and (ii) common deletion/reference fragment of unirradiated skin versus common deletion/reference fragment of irradiated skin after 5–16 mo after cessation of irradiation and thus differs from the shown values. (*A*) Representative agarose gel of PCR amplifications of the reference fragment. (*B*) Representative agarose gel of PCR products representing the common deletion. (*C*) Quantification with Storm 860 Phosphorimager. PCR products were separated electrophoretically and stained with Vistra Green dye for 16 h. Quantification based on green fluorescence of products was subsequently carried out by phosphorimager analysis. Values determined for PCR products representing the common deletion (b) were normalized to values of the reference fragments (a) of the same skin sample. (*D*) Representative result of real-time PCR for the common deletion. Results were calculated employing the $\Delta\Delta$CT method and are shown as factors of induction (For raw data see Table IV).

Table III. Differences of relative and dichotomised changes in the common deletion between baseline and follow-up measurements in irradiated samples when using real-time PCR

	Median relative difference	Interquartile range of differences	p-Value	Increase in common deletion (% soft criterion)	p-Value	Increase in common deletion (% strong criterion)	p-Value
Irradiated samples	+0.40	2.47	0.001	64.7	0.040	56.9	0.049
Unirradiated samples	+0.21	2.46	0.133	54.2	0.839	54.2	0.523

Explanation of criteria: see the section Statistical analysis; Values for "Increase in common deletion" of 50% indicate no change after irradiation. A value of 100% indicates perfect correlation between irradiation and *increase* of the common deletion. A value of 0% indicates perfect correlation between irradiation and *decrease* of the common deletion. Number of studied individuals n = 51.

Table IV. Real-time PCR raw data of 10 representative samples

					Ct values (mean of triplicates with standard deviation, SD)						
No.	Sex	Age	Skin type	Time point	IS	SD	CD	SD	ΔCt	ΔΔCt	Factor
1	F	24	III	No UVA week 0	19.24	0.11	35.93	0.18	17.41	0.00	1
				UVA week 2	18.91	0.16	32.30	0.56	16.63	−0.78	1.7
2	M	28	II	No UVA week 0	18.67	0.12	38.83	0.53	20.15	0.00	1
				UVA week 1	18.35	0.09	38.49	0.28	20.14	−0.02	1.0
3	M	30	III	No UVA week 0	18.73	0.07	37.51	0.11	18.78	0.00	1
				UVA week 1	18.70	0.07	37.33	0.15	18.63	−0.15	1.11
4	F	33	III	No UVA week 0	18.58	0.02	34.40	0.15	15.83	0.00	1
				UVA week 1	18.61	0.04	33.75	0.28	15.14	−0.69	1.6
5	F	18	II	No UVA week 0	18.33	0.06	34.00	0.23	15.17	0.00	1
				UVA week 1	19.52	0.16	33.52	0.16	14.00	−1.17	2.2
6	M	28	II	No UVA week 0	18.82	0.00	35.52	0.30	16.66	0.00	1
				UVA week 1	18.88	0.02	35.48	0.78	16.61	−0.06	1.0
7	F	18	III	No UVA week 0	17.73	0.29	36.72	0.37	18.99	0.00	1
				UVA week 1	17.31	0.08	34.66	0.20	17.35	−1.64	3.1
8	F	18	IV	No UVA week 0	17.53	0.17	36.69	0.34	19.15	0.00	1
				UVA week 1	17.93	0.09	35.62	0.08	17.69	−1.47	2.8
9	M	29	II	No UVA week 0	18.62	0.14	36.22	0.09	17.60	0.00	1
				UVA week 2	18.48	0.09	35.21	0.43	16.73	−0.86	1.8
10	F	20	II	No UVA week 0	18.96	0.07	37.61	0.51	18.65	0.00	1
				UVA week 1	17.74	0.19	36.54	0.28	18.80	0.15	0.90

respiratory chain (Wallace, 1999; Liu *et al,* 2002; Paradies *et al*, 2002) and, in a vicious cycle, inducing even more ROS and subsequently allowing mtDNA mutagenesis independent from the inducing agent (Nekhaeva *et al*, 2002). It is the characteristic of vicious cycles that they evolve at ever-increasing speeds. Therefore, the increase of the common deletion up to levels of 32-fold, independent of UV exposure, may represent the first *in vivo* evidence for the presence of such a vicious cycle. However, larger numbers of individuals need to be studied to confirm this possible long-term effect.

Relevance for photoaging It has been reported previously that urocanic acid (UCA) is a chromophore for UVA-induced photoaging (Hanson and Simon, 1998). Furthermore, UVA-irradiation of UCA has been shown to be capable of inducing the formation of (i) singlet oxygen and (ii) nicks in supercoiled plasmid DNA (Menon and Morrison, 2002). Together with this work, these reports strengthen the link between UVA-irradiation, strand breaks of circular mtDNA molecules as initiating damage to form large deletions (Shoffner *et al,* 1989; Berneburg *et al*, 1999), and the process of photoaging. Further studies are needed to unravel the underlying mechanisms regarding how the generation of mtDNA mutations by UVA-exposure actually translates into the morphologic alterations observed in photoaging of human skin.

Materials and Methods

Patients Fifty-two healthy human volunteers were enrolled after written informed consent. The age ranged from 18 to 35 yr, and all individuals were non-smokers and had no history of any severe skin disease, especially no photosensitivity disorders. Skin types ranged from Fitzpatrick type I to IV (Table III) and their buttock skin had not been exposed to natural or artificial UV radiation for a minimum of 1 yr.

UVA irradiation Experimental procedures were carried out after approval by the local ethics committee. Previously, sun-protected skin was exposed to UVA radiation from a Sellamed System

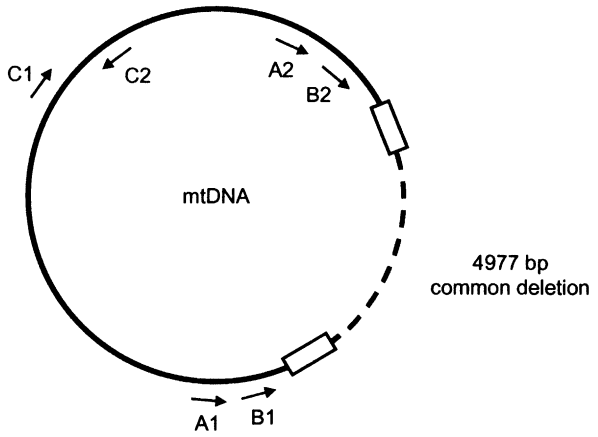

Figure 4
Schematic diagram of PCR primer positioning for detection of deleted mtDNA molecules: Arrows denote approximate primer binding sites. Primers A1/A2 and B1/B2 were chosen to allow nested PCR for confirmation of generated primary PCR products. Boxes designate deletion breakpoints. The distance between opposite primers is drastically reduced in molecules carrying the common deletion (dashed line). During DNA amplification, the polymerase extension time was designed to be too short for the amplification of wild-type PCR products only allowing the efficient amplification of the shorter and deleted mtDNA fragments. Primers C1/C2 were chosen to anneal in an area in which mutations of the mtDNA are not reported, thus serving as a reference fragment for the overall mtDNA population.

irradiation device (Sellas, Gevelsberg, Germany) with a UVA filter (Sellas, Gevelsberg, Germany) and an infrared absorbing filter (model UG1, Schott, Mainz, Germany) exclusively permitting wavelengths between 340 and 450 nm. The UVA irradiance was measured with a UVAMETER (Waldmann, Villingen-Schwenningen, Germany) and a photometer (IL 1700, International Light, Newburyport, MA, USA) and found to be 45 mW per cm^2 at body surface distance. Individuals were classified into Fitzpatrick skin types I–IV and subsequently irradiated with 15, 20, 25, and 30 J per cm^2, respectively, which led to a moderate erythemal response after the first week of irradiation. These UVA doses simulated standard UV- or sun-exposure conditions in the northern hemisphere during a holiday for 2 wk (Frederick and Alberts, 1992; Basu-Modak and Tyrrell, 1993).

In order to generate the common deletion, normal human buttock skin was irradiated 3 times daily, 5 consecutive days for week 1 and 2. If possible, before the beginning of irradiation (week 0) one biopsy as well as after week 1 and 2 of irradiation two punch biopsies were collected from exposed and unexposed skin areas, respectively. In the course of the study, however, not all 52 individuals consented to all samples being taken. Of the 52 individuals included in the study, 33 had biopsies taken at week 0 and 1, eight had biopsies taken at week 0 and 2, and seven had biopsies taken at week 0, 1, and 2. Four individuals had no biopsies taken at week 0 but at weeks 1 and 2. In these cases, biopsies taken from unirradiated skin sites were used as background to control for baseline levels of the common deletion. For real-time assessment of the last group, samples from one individual gave no PCR results, reducing the number of individuals investigated to three.

Nine individuals were biopsied at formerly irradiated and unirradiated skin sites 5–16 mo after cessation of exposure.

Split skin samples For separation of the dermis from the epidermis, skin samples were immersed in 1 M NaCl for 48 h at 4°C ($n = 5$).

DNA extraction Total cellular DNA was extracted from skin specimen employing the QIAamp DNA Mini Kit and the QIAamp DNA Blood Kit (Qiagen, Hilden, Germany).

PCR analysis

Nested-PCR PCR with primers C1 and C2 was carried out, amplifying a product of 247 base pairs in length. This region of mtDNA contains no reported mutations, thus representing the mt genome overall (Berneburg *et al*, 1997). Therefore, this PCR product served as a reference fragment during quantification. Amplification of fragments representing the common deletion was carried out as described previously (Henninger *et al*, 1993; Berneburg *et al*, 1997; Berneburg *et al*, 1999) employing published primer oligonucleotides (Anderson *et al*, 1981; Berneburg *et al*, 1999). In brief, oligonucleotides (A1/A2) were designed to anneal outside the 4977 bp common deletion (Fig 4). During DNA amplification, the polymerase extension time was designed to be too short for the amplification of wild-type PCR-products only allowing the efficient amplification of the shorter and deleted mtDNA fragments. To increase sensitivity and specificity a secondary, nested PCR was performed (B1/B2). For PCR, initial denaturation (94°C, 5 min) was followed by 28 cycles of denaturation (94°C, 1 min), annealing (58°C, 1 min), and extension (72°C, 25 s). A 2 μL aliquot of the primary PCR reaction was entered into reamplification (B1/B2) at an annealing temperature of 68°C, and a further decrease in the extension time to 15 s. PCR products were confirmed by restriction enzyme digest with Xba I (New England Biolabs GmbH, Schwalbach, Germany).

Quantification by Phosphorimager Amplification products were either visualized in a 1.5% agarose gel stained with ethidium bromide (0.25 μg per mL) or stained with Vistra Green dye (Molecular Dynamics, Heidelberg, Germany) for 16 h. Quantification based on green fluorescence of Vistra Green-stained PCR products was carried out by phosphorimager analysis (STORM 860, Molecular Dynamics, Heidelberg, Germany). Values determined for PCR products representing the common deletion were normalized to values of the reference fragments (see above) of the same skin sample. For long-term follow up of previously UV-exposed skin for up to 16 mo, expression of data as fold induction was calculated from two values: (i) common deletion/reference fragment of unirradiated skin versus common deletion/reference fragment of irradiated skin immediately after irradiation regimen and (ii) common deletion/reference fragment of unirradiated skin versus common deletion/reference fragment of irradiated skin 5–16 mo after cessation of irradiation.

Real-time PCR Real-time PCR was carried out as described previously (Koch *et al*, 2001) employing a TaqMan 7700 cycler (Applied Biosystems, Roth, Switzerland). In brief, amplification reactions were performed as 25 μL triplicates in a 96-well microplate format. Total mtDNA and deleted mtDNA reactions were carried out in separate tubes, each containing 100 ng DNA, 1 x TaqMan Universal PCR Master Mix, 300 μM dATP, dCTP, and dGTP, 600 μM dUTP, 300 μM of each IS primer, or 300 μM of each CD primer. The reactions were completed by adding 225 nM of the specific IS probe or CD probe. Primer oligonucleotides and probes for the common deletion (CD) as well as the housekeeping gene (IS) were employed identical to those published previously (Koch *et al*, 2001) and non-template controls showed undetectable fluorescence signals (ND). Carrying out real-time PCR up to 6 y after samples were initially raised, one individual failed to produce PCR products by this method, thus precluding this dropout from statistical analysis.

Statistical analysis Data analysis was performed using the statistical software SAS (version 8.2, SAS Institute, Cary, NC, USA). Since measurements in our longitudinal design were not complete for all time points, values of week 1 and 2 were averaged to avoid reduction of the sample size in a complete case analysis of the longitudinal data. The quantitative values for the common deletion are not directly comparable between individuals and were therefore transformed to a relative scale taking the baseline value before the irradiation regime as reference for each individual. In

addition to this quantitative approach, the effect of irradiation was also analysed using dichotomized response data using two dichotomization criteria in parallel: (i) the "strong" criterion considers an increase in the common deletion as present only when the baseline value is surpassed by at least 10% and (ii) using the "soft" criterion any numerical increase in the common deletion counts. In our paired design, the statistical comparison for the quantitative values was performed using the Wilcoxon signed-rank test, and the dichotomized response data were analysed using the sign test. p-values are reported as results of this statistical testing; those less than 0.05 were considered significant. The effect of the factors gender, age, and skin type on the dichotomized response was assessed in stratified contingency tables and jointly in a logistic regression model.

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