High Levels of Dipyrimidine Dimers Are Induced in Human Skin by Solar-simulating UV Radiation

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Abstract
UV light is considered an important contributor to skin cancer, but methods have been lacking to quantify specific UV-induced lesions in human skin in situ. We applied a newly developed 32P-postlabeling technique to measure specific UV-induced dipyrimidine dimers and 6-4 pyrimidine photoproducts, formed at most dipyrimidine combinations (4-6) and thought to be a major cause of lethal (7), mutagenic (8-il), and cancer, because mutations at dipyrimidine sites occur with high frequency in skin tumors (13, 14). In addition, the inability to repair dimers and cyclobutane dimers indicates a base sequence dependence of the repair process. The applied method has potential for the study of DNA repair as a determinant of individual susceptibility to skin cancer.

Introduction
Skin cancer is the most common form of cancer in humans. Epidemiological and experimental studies indicate that solar-induced UV radiation is a major cause of skin cancer in humans (1-3). UV irradiation induces many types of DNA damage including dipyrimidine lesions, cyclobutane dimers, and 6-4 photoproducts, formed at most dipyrimidine combinations (4-6) and thought to be a major cause of lethal (7), mutagenic (8-11), and transforming (12) events induced by UV exposure. Dipyrimidine lesions are likely to be important in the pathogenesis of skin cancer, because mutations at dipyrimidine sites occur with high frequency in skin tumors (13, 14). In addition, the inability to efficiently repair pyrimidine dimers in xeroderma pigmentosum renders these patients hypersensitive to sunlight and to the development of multiple skin tumors (15, 16). Evidence from sporadic skin cancers also suggests that DNA repair rates relate to skin cancer susceptibility (17-19).

Although several methods are available to measure pyrimidine dimers, they have severe limitations regarding sensitivity at the level needed for human samples in situ and specificity needed to distinguish the different dipyrimidine combinations of cyclobutane and 6-4 photoproducts. In the present study, we applied a novel 32P-postlabeling technique (20, 21) for the detection of dipyrimidine lesions in humans. Two specific cyclobutane dimers and one 6-4 photoproduct were measured in human skin biopsies after exposure to physiologically relevant doses of SS-UV. Furthermore, we describe the repair kinetics of these lesions after irradiation. The results indicate that SS-UV radiation induces levels of DNA damage far exceeding any other known external exposure. The method may provide a useful in vivo screening test of DNA repair.

Materials and Methods
Volunteers. Sixteen volunteers (11 females and 7 males) were recruited from Turku medical students and laboratory staff. The study plan was approved by the local ethical committee of the University of Turku, Finland, and the volunteers gave their informed consent to participate. Exclusion criteria included a history of exposure to sunlight or artificial UV sources within the past 6 months, the presence of skin diseases, and any abnormal sensitivity to sunlight. The ages of the volunteers varied from 18-49 years (median age, 24 years).

UV Radiation and Collection of Biopsies. A Philips HP 3136 sun lamp was used for the irradiation of 1-cm2 areas of buttock skin. The spectral irradiance of this lamp closely mimics the solar spectrum throughout the UV range (22), as determined with a double-grating radiometer Optronic 742 with Teflon diffuser input optics and calibrated against a 1000 W halogen standard lamp traceable to the United States National Institute of Standards and Technology. The administered doses were given as their erythemally corrected values, using the Commission Internationale de L'Eclairage weighting spectrum (23), and were 0, 50, 100, 200, and 400 J/m2 in eight volunteers (photoproduct induction study) and 400 J/m2 in six volunteers (photoproduct repair study). In the photoproduct induction study, five 3-mm punch biopsies from the irradiated skin sites and from the nonirradiated control site were taken 10 min after irradiation, whereas in the photoproduct repair study, six biopsies were taken at 0, 1, 3, 24, and 48 h after irradiation and from the nonirradiated control site. All samples were coded, and the rest of the work was performed in a blind fashion.

DNA Extraction and Photoproduct Analysis. Biopsies were immediately frozen at -20°C after sampling. For DNA isolation, tissue samples were frozen in liquid nitrogen, and epidermis was dissected from dermis. Epidermis was homogenized in 50 mM Tris-HCl (pH 8.0) containing 1% SDS and 500 µg/ml proteinase K and incubated at 37°C overnight. Samples were extracted with chloroform-isoamyl alcohol twice, precipitated with ice-cold et-
anol, washed with 70% ethanol, and dried in a Speed-Vac vacuum centrifuge. Samples were redissolved in 50 mM Tris-HCl (pH 7.4) and incubated with 10 μg/ml RNase A and 5 units/ml RNase T1 at 37°C for 2 h followed by a chloroform-isooamyl alcohol extraction. After that, DNA was precipitated by ice-cold ethanol, washed with 70% ethanol, and dried in a Speed-Vac vacuum centrifuge. From each 3-mm punch biopsy, we could isolate 3–4 μg of DNA. For each 32P-postlabeling assay, 2.5 μg of DNA were digested (Fig. 1; Ref. 20). DNA was dissolved in 10 mM Tris-HCl (pH 7.5) and 5 mM MgCl2. Snake venom phosphodiesterase was added at 1 milliunit/nanomole of nucleotides and incubated for 20 h at 37°C. An equal volume of 60 mM sodium acetate and 0.1 mM zinc chloride (pH 5.0) was added. pH was adjusted with HCl to 5.0, and samples were incubated for 4 h at 37°C with 5 milliunits of prostatic acid phosphatase/nanomole of nucleotides. After incubation, pH was adjusted to 8.0, proteinase K was added up to a concentration of 125 μg/ml, and the samples were incubated for 90 min at 37°C. Proteins were precipitated with ice-cold ethanol by vortex mixing followed by centrifugation at 14,000 rpm for 15 min. The samples were dried in a Speed-Vac vacuum centrifuge. The samples were phosphorylated in a total volume of 2 μl containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 10 mM 2-mercaptoethanol, 1.3 pmol of [γ-32P]ATP (3,000 Ci/mmole), and 6 units of T4 polynucleotide kinase. Incubations were performed at 37°C for 1 h.

The HPLC assay was performed on a Beckman instrument operated with System Gold software and coupled to radioisotope detector module 171. A Kromasil C18 column, manufactured by Phenomenex (2 × 250 mm; 5-μm particle size), with a precolumn filter was used. The labeled products were diluted with water up to 10 μl and injected onto the HPLC. Separation of photoproducts was performed using gradient elution with a buffer [0.5 M ammonium formate and 20 mM orthophosphoric acid (pH 4.6)] that was mixed with methanol. The initial elution was isocratic with 100% buffer for 2.5 min, followed by a switch to 0.5 M ammonium formate, 20 mM orthophosphoric acid, and 0.1% triethylamine (pH 7.4). At 50 min, a linear methanol gradient (0–5%) was pumped for 40 min, followed by an increase to 100% methanol over 10 min. The column was washed for 10 min with methanol, followed by gradient to 100% water for 7 min, and was finally switched back to the initial buffer. The flow rate was 0.21 ml/min. The products were detected by a Beckman 32P radioisotope detector. For calculations of product yields, the HPLC peaks areas were integrated with Beckman System Gold software. Radioactivity from the unirradiated control biopsy was subtracted as background. Each sample was analyzed on two to times.

Photoproduct standards were synthesized from nonmodified trinucleotides (24). The standards of measuring photoproducts were mixed with hydrolyzed nonmodified DNA and labeled together with the human samples. The quantification took into account the amount of a standard added and a peak area obtained from human samples. The labeling efficiency at the conditions used varied (~100% for TT=T, 80% for TT=C, and 30% for TT=C; Refs. 20, 21, and 24).

Results
The DNA digestion procedure released cyclobutane dimers and 6–4 photoproducts as trinucleotides with a nonmodified nucleotide at the 5' end of the photoproduct, which guaranteed efficient labeling by polynucleotide kinase (20, 21). In the HPLC analysis, synthetic standards were used to identify the photoproducts (20, 21). The migration of two cyclobutane dimers, TT=T and TT=C, and one 6–4 photoproduct, TT=C, is shown in Fig. 2B in a biopsy obtained after a SS-UV dose of 400 J/m2.

The levels of photoproducts varied extensively between the volunteers given equal doses (Fig. 3). The individual data on six volunteers on the induction of the cyclobutane dimers at a dose of 400 J/m2 are shown in Fig. 2. The levels of cyclobutane dimers were: TT=T, 1.1–5.9 photoproducts/106 nucleotides and TT=C, 0.8–7.0 photoproducts/106 nucleotides.

The mean levels of the three photoproducts in biopsies from eight individuals irradiated with doses of 50–400 J/m2 are shown in Fig. 4. The three lesions were detected even at 50 J/m2, the smallest dose used. Photoproduct formation increased linearly with dose in the following order: TT=C = TT=T > TT=C. At 400 J/m2, the highest dose applied, the adduct levels were: TT=T, 2.3 ± 0.7 photoproducts/106 nucleotides; TT=C, 2.3 ± 0.8 photoproducts/106 nucleotides; and TT=C, 0.4 ± 0.2 photoproducts/106 nucleotides.

We were able to follow the DNA repair kinetics of the two cyclobutane dimers, TT=T and TT=C, as shown in Fig. 5. These data points are the average values obtained from six individuals. The repair of TT=T apparently took place in two phases, whereas TT=C followed one-phase repair kinetics without a lag-phase in the beginning. After 48 h, 68% of TT=T and 85% of TT=C were repaired.

Discussion
The advantages of the present 32P-postlabeling method over previous methods (17, 25–27) are high sensitivity, requirement of a small amount of DNA, and the possibility of studying different photoproducts in different dipyrimidine pairs in one sample (27). The combination of the 32P-postlabeling assay with HPLC analysis provides a powerful tool in terms of sensitivity, resolution, reproducibility, and quantitation for DNA adduct determination (21, 28–30). SS-UV irradiation in vivo resulted in a linear induction
of cyclobutane dimers and 6–4 photoproducts. The combined level of the cyclobutane dimers TT=T and TT=C in human skin after 400 J/m² is around 5 photoproducts/10⁶ nucleotides. This is still an underestimation, because no provision is made for the presence of C=T and C=C cyclobutane dimers (21). A normal nucleotide at the 5' side of photoproducts can be any one of four. Taking this into the consideration, these cyclobutane dimers can be calculated to amount to some 20 photoproducts/10⁶ nucleotides at 400 J/m². The previous estimations of the level of cyclobutane dimers in human DNA differ widely (26, 31), and expert reviewers of the topic refrain from citing such values (5, 32). The SS-UV erythema threshold (minimal erythema dose) for the Finnish skin type using the light source in this study is between 280 and 570 J/m², based on the large material of the Dermatology Department in Turku (23). The SS-UV dose of 400 J/m² used in this study should have caused an erythemal response in some of the subjects.

Photoproduct levels can be compared to those of other exogenous DNA adducts reported in human tissues (Table 1). All of the agents listed are known or suspected human carcinogens at organs where adduct levels have been measured. The data have been collected from different studies, and no consideration is taken for variables such as duration

Table 1 DNA adducts induced by specific carcinogenic exposures in human target tissues

<table>
<thead>
<tr>
<th>Adduct</th>
<th>Tissue</th>
<th>Level (per 10⁶ bases)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-meG (Ref. 33)</td>
<td>Lung</td>
<td>100</td>
<td>Smoking</td>
</tr>
<tr>
<td>BP (Ref. 34)</td>
<td>Lung</td>
<td>10</td>
<td>Smoking</td>
</tr>
<tr>
<td>Benzidine-G (Ref. 35)</td>
<td>Urothelium</td>
<td>3</td>
<td>Occupation</td>
</tr>
<tr>
<td>PhiP-G (Ref. 34)</td>
<td>Colon</td>
<td>3</td>
<td>Roasted meat</td>
</tr>
<tr>
<td>Tamoxifen (Ref. 30)</td>
<td>Endometrium</td>
<td>0.3</td>
<td>Drug</td>
</tr>
<tr>
<td>T = T and T = C⁴</td>
<td>Skin</td>
<td>2000</td>
<td>SS-UV, 400 J/m²</td>
</tr>
</tbody>
</table>

⁴ Present study (all four different trinucleotides).
of exposure. These data show that photoproducts are by far more abundant than other quantified DNA adducts formed by exogenous agents. However, different types of endogenous adducts, such as oxidized base modifications, are present at high levels in human DNA. UV radiation is able to induce reactive oxygen species such as superoxide anion, singlet oxygen, and hydrogen peroxide. However, the role of these photoproducts in UV carcinogenesis is unknown (15). Xeroderma pigmentosum patients have ~1000 times higher risk of developing skin cancer than that expected for the general population, but they do not have a proportional increase in internal cancers (16). The extraordinarily high extent of DNA damage induced by solar irradiation may be the main explanation for the high prevalence of skin cancers in xeroderma patients who are unable to repair the damage.

The present method can also be used to study DNA repair rates, as was shown. The rates of TT=T and TT=C repair were somewhat different, with 50% being repaired in approximately 32 and 10 h, respectively. Our data suggest that the repair rates of cyclobutane dimers depend on the dipyrimidine pair forming the lesion, because they are known to depend on the sequence context in DNA (32).

Our data demonstrate extensive photoproduct induction in human skin by small doses of SS-UV. Such high DNA modification levels, exceeding by orders of magnitude the adduct levels induced by any other studied exogenous carcinogens, may be one reason for the well-documented effects of exposure to sunlight on human carcinogenesis.

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References


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