PCR-Based Methodology for the Determination of the Photoprotection Afforded by Sunscreen Application

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ABSTRACT

We have applied a PCR-based methodology to study the DNA damage induced by UV-A, UV-B and sunlight itself. Our results, employing a cell-free system, indicate that UV-B (310 nm) is approximately 30-fold more potent at inhibiting DNA synthesis than UV-A (365 nm). We were also able to show that 20 min of sunlight exposure on a summer day induced DNA damage capable of inhibiting DNA synthesis. Hence, this methodology has a sensitivity suitable to detect biologically relevant doses of UV light. In addition, we propose that this technique may be suitable to assess the relative photoprotection of commercially available sunscreens. We present here preliminary data on the photoprotection afforded by the topical application of sunscreen. This photoprotection was measured by a reduction in the subsequent UV-B- and UV-A-induced DNA damage when sunscreen was applied. Our results demonstrate that the particular sunscreen tested was effective against both UV-B and UV-A. However, the estimated photoprotective factor of the sunscreen (against both UV-A and UV-B) was approximately tenfold less than the stated Sun Protection Factor (SPF) of 25. This methodology may also be useful in identifying new photoprotective agents by assessing their relative value as UV-B and UV-A absorbing agents.

INTRODUCTION

Sunlight exposure is known to be potentially carcinogenic, and excessive sun exposure is responsible for the increasing incidences of skin cancer. Skin cancer rates have been shown to be rising at a rate second only to those of lung cancer (8), with an estimated 2 million nonmelanoma skin cancers reported annually worldwide (17). The rapid rise in the incidences of skin cancer in Western populations can be partly attributed to the changing lifestyles since the war, where deliberate sun exposure has increased sharply (11).

Sunlight is comprised of two forms of UV light: UV-A (315–400 nm) and UV-B (280–315 nm), both of which have been implicated in carcinogenesis (3,15). Sunlight (and the UV light components therein) induce skin cancer by damaging DNA. The main types of DNA damage induced by UV-B are cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4-pps). UV-A is a weaker inducer of CPDs and 6-4-pps and is thought to induce DNA-damage via an oxidative pathway (1). UV-A represents 95% of solar UV light, with UV-B representing 5% of solar UV light (8). The lower wavelength UV-B is more potent at damaging DNA than UV-A, while UV-C, the most potent form of DNA-damaging UV light (100–280 nm), is currently absorbed by the atmosphere (including the ozone layer). Sunlight UV-B intensity is measured using minimal erythema doses (MED), which is defined as the dose required to induce erythema (sunburn) in human skin (8). It

is important to note that the skin penetration depth of UV light is inversely proportional to its DNA reactivity (i.e., UV-A penetrates more deeply than UV-B). This difference is significant in terms of the location of the biological effect of UV-A and UV-B.

Given the role of UV light in skin cancer, it has been an important goal to develop methodologies suitable for studying UV-induced DNA damage. We have recently described a methodology for the sensitive detection of DNA-damaging agents (9). The method is based the well-known effect whereby DNA-damaging lesions inhibit DNA synthesis (7,10,13). Thus, damaged DNA is a poor substrate for PCR; therefore, UV-induced damage can be detected by a corresponding reduction in PCR amplification following UV exposure. Increasing exposure to UV light leads to increased damage accumulation and a dose-dependent decrease in PCR amplification. Our methodology uses an immobilized 1.7-kb DNA target, which is subject to DNA damage induction in vitro. In this paper, we demonstrate the use of this methodology in studying DNA damage induced by sunlight and its principal UV components. This method benefits from detecting a range of damage types that are capable of blocking DNA synthesis. We chose to study the DNA damage induced in the mammalian *p53* gene because of the implication of this gene in skin cancer (1), although any target gene would presumably yield valuable data on DNA synthesis inhibition.

In addition to determining UV-in-

Table 1. PCR Primers Used in the Polymerase Inhibition Assay

PCR Target	Forward Sequence	Reverse Sequence	Size
Mouse <i>p53</i>	Bgaggagtttttgaaggccc	ccctacctcactacaggtgacc	1686 bp
Nested primers	cctgcccagccactccatg	acacctgctggagctgtgggg	1542 bp

PCR primer sequences for amplification of the 1.7-Kb target and the nested re-PCR after UV treatment.

B = biotin molecule.

duced DNA damage, we present data on the protective effect afforded by the topical application of sunscreen. To assess the usefulness of sunscreens in absorbing UV light, we applied sunscreen to an artificial skin substrate covering our DNA target, as previously described (5). We then determined the level of inhibition of DNA synthesis in samples with and without topical sunscreen application to ascertain the UV-absorbing potential of the sunscreen. We propose that this methodology may have value in describing the relative photoprotection of a range of sunscreens and sunscreen components. Additionally, this methodology may allow the identification of new photoprotective agents.

MATERIALS AND METHODS

Preparation of the Target PCR Product

The PCR target was amplified from mouse genomic DNA (Promega, Southampton, UK) yielding a 1.7-kb region for DNA damage induction, as previously described (9). The primers employed were obtained from Cruachem Ltd. (Glasgow, UK) and are shown in Table 1.

Immobilization of the Target PCR Product

The target PCR product was quantitated by spectrophotometry at 260 nm and was diluted to 40 pgs/ μ L. This approximates to 10^7 copies/ μ L of a 1700-bp DNA fragment. Eighty picograms of PCR product (2×10^7 copies) were immobilized in each well of a streptavidin-coated microplate (Advanced Biotechnologies, Epsom, UK) by virtue

of the use of a biotinylated primer in the first PCR amplification. The immobilization was performed by incubating 80 pg PCR product (in 20 μ L) with an equal volume of binding and washing buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA and 2 M NaCl) for 1 h at room temperature. The immobilization was performed as a master mixture, with equal volumes of the PCR product/binding and washing solution aliquoted into each well to ensure an even distribution of the PCR targets. After immobilization, 10 μ L TE buffer was added to the bound PCR products. This allowed the production of some oxidative free radicals by the incident UV light.

Nested Re-PCR Posttreatment

The UV-treated PCR targets were re-amplified in the microplates, using nested primers (Cruachem Ltd.) shown in Table 1, giving a nested product of 1542 bp, as previously described (9). This nested amplification identifies the extent of DNA damage induced in the DNA target. Hence, it is imperative that this amplification is performed within the exponential phase. Extensive calibration experiments were performed to ensure that such quantitative conditions were maintained. The nested PCR was performed with 1 U Extensor DNA polymerase (Advanced Biotechnologies), 50 μ M each dNTP, 1 mM $MgCl_2$, 80 pg target DNA in 50 μ L 1 $\times$ Extensor polymerase buffer (Advanced Biotechnologies). The nested PCR was performed for 19 cycles in a PTC-200TM DNA engine (MJ Research, Warrington, MA, USA).

Assessment of Polymerase Inhibition

The re-amplified nested PCR products were electrophoresed on 1% aga-

rose gels and stained with SYBRGold[®] (Molecular Probes, Leiden, The Netherlands). The fluorescent band intensities were analyzed using the Quantity One software of the Gel-DocTM 2000 image analysis equipment (Bio-Rad Laboratories, Hemel Hempstead, UK). Relative PCR amplifications of the damaged samples were calculated based on the amplification of the undamaged control, which was assigned an amplification factor of 100%, using the equation taken from Kalinowski et al. (10): $A_D = I_D/I_0 \times 100\%$ where A_D = relative amplification of damaged sample, I_D = intensity of PCR band of damaged sample and I_0 = intensity of PCR band of undamaged sample.

DNA synthesis inhibition experiments were performed independently in triplicate; the average relative PCR intensities were calculated and plotted on graphs of relative PCR against dose, including standard errors. The graphs were plotted in Microsoft[®] Excel[®], and trendlines were added to the points using the same software.

UV Light Treatments

Immobilized target PCR products (80 pg, 2×10^7 copies of the target) were exposed to varying amounts of UV-B (310 nm) and UV-A (365 nm) light. The UV-B light was administered from a Syngene (Genetic Research Instrumentation, Essex, UK) GLM-6 lamp (6 W) at a calibrated dose rate of 50 J/M²/s. The UV-A light was administered from a Syngene GLL-4 lamp (4 W) at a calibrated dose rate of 6 J/M²/s. The dose rates were estimated using a UV-X UV dosimeter (UVP, Cambridge, UK) using sensors for UV-A (365 nm) and UV-B (310 nm). No external filters were used to cut out potential contaminating shorter wavelengths. However, each lamp had integral filters that blocked lower wavelength light. Hence, no UV-C light was emitted from the UV-B lamp, and no UV-B light was emitted from the UV-A lamp.

In the case of sunlight exposures, the DNA targets were exposed to sunlight during the hottest part of the day (11 am to 2 pm) for the appropriate amount of time. Sunlight exposures were performed on clear summer days when temperatures exceeded 30°C.

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Evaluation of the Photoprotective Effect of Sunscreen

In the experiments to assess the role of sunscreen in UV-induced DNA damage formation, a commercially available factor 25 sunscreen was employed. The sunscreen (20 mg) was applied to 7 cm² artificial skin substrate (approximately 3 mg/cm²) (transpore tape; 3M Company, St. Paul, MN, USA) (5) before UV exposure. This piece of tape covered each strip of the microplate containing 16 wells. The same amount of sunscreen was applied to the piece of tape covering each strip of the microplate. This was ensured by weighing the sunscreen after application and by using the same size piece of tape to cover the microplate. The tape contains a grooved patterned surface and thus mimics human skin (5). Wiping the sunscreen over the surface of the tape (with a gloved finger) effectively filled up the grooves and distributed the sunscreen evenly. It is important to note that the samples exposed to UV light, without sunscreen, were also covered by the transpore tape. After exposing the DNA target to UV light, in the presence or absence of sunscreen, DNA synthesis inhibition was measured to determine the photoprotective effect of the sunscreen. The UV light sources were used in this case rather than using actual sunlight exposure because of the

inherent variation in sunlight levels. The doses of UV-B necessary to inhibit DNA synthesis were greater than in previous experiments, presumably because of the preferential shielding of the DNA target from the lower wavelengths by the artificial skin substrate. The UV dose reaching the immobilized target was measured by covering the sensor with the transpore tape.

RESULTS

Detection of UV-B-Induced DNA Damage

Figure 1 shows the DNA damage introduced by the irradiation of the DNA target with UV-B light (310 nm). It can be seen from Figure 1 that doses as low as 500 J/M² were capable of causing DNA damage that inhibits DNA synthesis. This dose corresponds to the 2000–3000 J/M² necessary to induce visible toxicity to primary human foreskin fibroblasts (results not shown) emphasising the sensitivity of this assay. The level of inhibition of DNA synthesis increased with increasing UV-B dose. At the higher UV-B doses, PCR amplification was reduced to 20%. The fact that total DNA synthesis inhibition was not achieved was presumably the result of either (i) the presence of undamaged strands after UV treatment due to shield-

ing, (ii) the background fluorescence in the gel and (iii) the occasional bypass of the DNA damage by the polymerase.

The doses of UV-B shown to be detectable in this study are within the dose range shown to induce erythema in human skin. The MED values range from 200 to 1000 J/M² depending on skin type (8). Therefore, this methodology appears to be sensitive to doses of UV-B within the biologically relevant dose range.

Detection of UV-A-Induced DNA Damage

Figure 2 shows the inhibition of DNA synthesis induced by exposure to UV-A light (365 nm). Figure 2 demonstrates that approximately 15 kJ/M² of UV-A were required to be detectable by the inhibition of DNA synthesis. Therefore, by comparing effective doses of UV-B and UV-A, we can estimate that UV-B is 30-fold more DNA damaging than UV-A. No visible toxicity was observed by exposing human primary foreskin fibroblasts to doses exceeding 25 kJ/M² (results not shown), again emphasizing the sensitivity of this PCR-based assay. It was not possible to determine the toxic dose range of UV-A light because of the long exposure times necessary to expose the cells to high doses of UV-A light and the adverse effect this had on the cells, which

Figure 1. UV-B-induced DNA synthesis inhibition, taken from Jenkins et al. (9). Immobilized DNA exposed to a range of UV-B doses was quantitatively amplified by PCR. At higher doses of UV-B, DNA damage is detected by a reduction in PCR amplification.

Figure 2. UV-A-induced DNA synthesis inhibition. Immobilized DNA exposed to a range of UV-A doses was quantitatively amplified by PCR. At higher doses of UV-A, DNA damage is detected by a reduction in PCR amplification.

were dosed in a minimal amount of culture media to prevent the UV absorbance of the media.

Detection of Sunlight-Induced DNA Damage

After demonstrating that UV-B and UV-A were both capable of inhibiting DNA synthesis, we assessed the DNA damage induced by direct sunlight exposure to test the sensitivity of the method with biologically relevant doses of UV light. Figure 3 shows the results obtained and demonstrates that sunlight exposure of 20 min was sufficient to induce detectable levels of DNA damage. The inhibition of DNA synthesis increased with increasing exposure to sunlight, dropping to 25% amplification after 2 h sunlight exposure.

Determination of the Photoprotective Effect of Topical Sunscreens

To demonstrate the photoprotective effect of UV absorbing sunscreens against UV-induced DNA damage, we employed this PCR-based methodology to determine sunscreen effectiveness. UV light was administered to samples with and without sunscreen, but each

bearing the artificial skin substitute (transpore tape). Figure 4 shows the effect of UV-B exposure on the inhibition of DNA synthesis with and without the sunscreen present. Figure 5 shows the same result achieved with UV-A light. As can be seen from Figures 4 and 5, the sunscreen protects DNA from DNA damage, as is demonstrated by the greater inhibition of DNA synthesis evident in the samples that did not have sunscreen applied. This photoprotection, however, is substantially less than the stated Sun Protection Factor (SPF) of 25 of this sunscreen. Indeed, the maximum photoprotection conferred (the difference in DNA damage induction with and without sunscreen) appears to be approximately two- to three-fold. It is interesting to note from comparing Figures 1 and 4 that more incident UV-B light was needed when the tape covered the immobilized DNA to inhibit DNA synthesis. Because the UV meter readings were adjusted by covering the sensor with the tape to ensure similar exposure levels, this may indicate that the tape cuts out the more damaging lower wavelength UV-B light preferentially. In contrast, by comparing Figures 2 and 5, the tape appears transparent to UV-A.

Figure 3. Sunlight-induced DNA synthesis inhibition. Immobilized DNA exposed to a range of sunlight exposures was quantitatively amplified by PCR. At higher sunlight levels, DNA damage is detected by a reduction in PCR amplification.

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DISCUSSION

We describe here a rapid PCR-based methodology for the detection of DNA damage in a reporter target gene exposed to the component forms of solar UV light and sunlight itself. Given that DNA damage is a known precursor of carcinogenesis and sunlight is a known human carcinogen (8), this methodology may allow the assessment of carcinogenic risk using a relevant biological marker (e.g., DNA). Monitoring the solar UV content may become necessary, if the current trend towards ozone thinning continues. It is well known that any decrease in ozone thickness would substantially increase our exposure to UV light (8,15), perhaps including exposure to the more potent UV-C wavelengths (100–280 nm). Increases in the UV content of solar light would inevitably increase the incidences of skin cancer.

The laboratory-based studies using UV-A and UV-B lamps demonstrated the detection of DNA damage induced by both wavelengths through the inhibition of DNA synthesis. These results showed that UV-B was approximately 30-fold more potent at DNA damage induction than the longer wavelength UV-A (inhibition of DNA synthesis at 500 J/M² compared to 15 kJ/M²). To demonstrate that the method was capable of detecting biologically relevant levels of UV light-induced DNA damage, sunlight exposures were assessed for DNA synthesis inhibition. The

method was shown to be capable of detecting sunlight-induced DNA damage after 20 min sunlight exposure. The sensitivity of our methodology in detecting UV-B- and UV-A-induced DNA damage compared fairly well to the cellular toxicity assessments performed in our laboratory and to several other techniques applied to monitor UV DNA damage levels. It has been reported that *p53* mutations can be detected after exposure to 500–1000 J/M² UV-B (18). In addition, it has been shown that 500 J/M² (4) to 900 J/M² (14) of UV-B induce detectable levels of DNA damage in the comet assay. The corresponding dose of UV-A, detectable with the comet assay, has been shown to be 9000 J/M² (14). However, the induction of *p53* expression has been shown to occur following doses of 40–140 J/M² UV-B (2,12), which were somewhat lower than those detectable here. Hence, *p53* induction may be a more sensitive indicator of UV light exposure than the induction of DNA damage. However, this methodology allows the rapid high-throughput assessment of UV exposure through the assessment of DNA damage induction, which is an essential endpoint in UV light-induced skin cancer.

We have shown previously (9) that UV-C inhibits DNA synthesis at doses as low as 20 J/M², whereas the effective doses of UV-B and UV-A are 500 and 15 000 J/M², respectively. This comparative dose response data supports the well-known fact that UV-B

and UV-C are more potent DNA-damaging agents than UV-A (6). The difference in effective dose levels between the UV subtypes is perhaps related to the different damage types induced by the different wavelengths. UV-C and UV-B mainly induce pyrimidine dimers and 6-4-pps, whereas UV-A is thought to induce oxidative DNA damage (1). The UV-A-induced DNA damage certainly appears to be less DNA synthesis inhibiting and therefore less detectable in this study. However, that is not to say that UV-A does not contribute to sunlight-induced skin cancer. In our experiments, UV-A has reduced reactive oxygen species (ROS)-generating potential, as the chromophores and transition metal ions necessary for ROS-generation are not as abundant here as in vivo. The contribution of different DNA damage types (e.g., strand breaks) to the DNA synthesis inhibition reported here are unknown because the specific lesion types induced by the various UV sources were not detectable with this methodology.

We also demonstrate here that this methodology allows the rapid measurement of the photoprotection of sunscreens against sunlight-induced DNA damage. We have shown here that topical sunscreen application can ameliorate the DNA damage induced by UV-B and UV-A. In both cases of UV exposure, the reduction in DNA damage due to the protective sunscreen was approximately tenfold less than the SPF value

Figure 4. Measurement of sunscreen photoprotection against UV-B light (SPF = 25). A similar experiment to that shown in Figure 1, except UV-B induced DNA damage was measured in the presence or absence of sunscreen. The difference in the curves shows the photoprotection provided by sunscreen.

Figure 5. Measurement of sunscreen photoprotection against UV-A light (SPF = 25). A similar experiment to that shown in Figure 2, except UV-A-induced DNA damage was measured in the presence or absence of sunscreen. The difference in the curves shows the photoprotection provided by sunscreen.

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of 25. It could be interpreted from these results that SPF values underestimate the potential DNA-damaging effects of sunlight; subsequently, we may be overestimating their photoprotective potential. However, our experimental design using DNA damage to monitor UV levels differs from that used to calculate SPF values, which are based on reductions in the dose-inducing erythema. In addition, at high doses of UV-A and UV-B, the exposure times exceed 1 h, and there is a possibility that evaporation of the sunscreen may alter its photoprotection. Further experiments with other sunscreen products are also necessary to confirm the findings reported here. Future investigations based on the photoprotective effect of a range of sunscreens to sunlight rather than laboratory-based UV lamps may yield important information on the effectiveness of sunscreens in absorbing solar UV light. In particular, employing sunlight rather than artificial UV sources would allow the study of biologically relevant doses of UV light rather than extrapolation

from high doses, as has been necessary here. In addition, the effect of the re-application of the sunscreens can be tested to investigate the effective half-lives of the sunscreens used.

Similar experiments to those reported here on the UV-absorption of sunscreens could be used to assess the relative UV-absorbing potential of new sunscreen components and may allow the high-throughput identification of new photoprotective agents that may be suitable for inclusion in sunscreens. A recent report has investigated the inclusion of DNA repair enzymes in sunscreens (16). Our methodology may be well suited in validating the value of such sunscreens in reversing the accumulation of sunlight-induced DNA damage. The most important feature of this PCR-based methodology is that it actually measures UV light exposure by measuring the amount of DNA damage induced. The detection of such DNA damage is the ultimate endpoint for any system monitoring human UV exposure.

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