The Multilayered Organization of Engineered Human Skin Does Not Influence the Formation of Sunlight-induced Cyclobutane Pyrimidine Dimers in Cellular DNA

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Abstract

Solar UVB initiates skin cancer mainly by generating highly premutagenic cyclobutane pyrimidine dimers (CPDs) and subsequent mutations in critical growth control genes. It is universally presumed that the upper epidermis in human skin blocks a significant portion of the incident UVB, thereby protecting the cancer-prone basal layer from CPD formation. Using two sensitive techniques for measuring CPD in cellular DNA, we confirmed that the multilayered organization of engineered human skin efficiently shields the basal layer against 254-nm UVC (which is not present in terrestrial sunlight) but, very unexpectedly, provides virtually no protection against environmentally relevant UVB. This underscores the importance of regular sunscreen use, which, in light of our data, may constitute a considerably more important first line of defense against photocarcinogenesis than previously believed.

Introduction

The induction of CPDs in DNA by the UVB component of sunlight is a preeminent determinant in the development of skin cancer, the most frequent neoplasia in Caucasian populations (1, 2). The initiation of most cutaneous tumors requires penetration of solar UV to the actively dividing (i.e., cancer-prone) basal layer of the epidermis, where CPD and subsequent mutations are generated in oncogenes and tumor suppressor genes, including the p53 tumor suppressor gene, which is mutated specifically by sunlight in >90% of nonmelanoma tumors (3–6). Basic scientific intuition dictates that the upper epidermal layers, most prominently the stratum corneum, passively protect the basal layer against CPD formation (and, hence, against sunlight-induced mutation and cancer) by scattering and absorbing a significant portion of the incident UVB (7). Here, we have used EHS tissue in addition to pure human keratinocyte or fibroblast monolayer cultures that were irradiated with environmentally relevant UVB (290–320 nm) or broad-spectrum SSL (290–1100 nm) as well as with the model mutagen 254-nm UVC (which is vastly attenuated at the surface of the earth by stratospheric ozone). For each experimental condition, CPD formation was quantified at two levels: (a) within the genome overall (hereafter referred to as “global CPD frequency”) and (b) at nucleotide resolution (hereafter referred to as “CPD distribution”) along exons 5–8 of the p53 tumor suppressor gene. This comparative analysis, i.e., using different UV wavelengths in conjunction with diverse model systems, has permitted a direct evaluation of the UV shielding capacity of the upper epidermis. Our results indicate that the multilayered organization of cutaneous cells in the upper epidermis of human skin does not attenuate the formation of CPD induced by environmentally relevant solar wavelengths in the basal layer, thus bringing into question a universally accepted maxim in the field of photocarcinogenesis.

Materials and Methods

Cutaneous Cell Isolation. Keratinocytes and fibroblasts were isolated from normal human skin biopsies as described previously (8, 9). Briefly, fresh skin biopsies were immediately cut into 5-mm × 5-mm pieces and treated with 500 mg/ml thermolysin (Sigma Chemical Co., St. Louis, MO) overnight at 4°C, which allows complete separation of the epidermis from the dermis. The epidermis was treated with 0.05% trypsin-0.1% EDTA, and the dermis was treated with collagenase (0.1%). The cells were then treated separately and adjusted to the desired concentration.

Keratinocyte and Fibroblast Cultures. Both cell types were seeded into culture flasks (Falcon, Becton Dickinson, Lincoln Park, NJ). Keratinocytes were seeded at a concentration of 9 × 10^4 cells/flask in growth-factor-supplemented DMEM. The medium was changed three times a week for both cell types. When the cultures reached 70–80% confluence for keratinocytes and 100% for fibroblasts, the cells were detached with trypsin-EDTA solution, washed twice, and then resuspended in DMEM or DMEM-supplemented medium for human skin preparation.

EHS Preparation. EHS was produced as described previously (9, 10). Briefly, bovine type I collagen (2 mg/ml) was mixed with a normal human fibroblast suspension (1.5 × 10^6 cells) and poured into a Petri dish (35-mm diameter) to produce dermis. These tissues were cultured in 10% FCS-supplemented culture medium for 4 days, and then keratinocytes (9 × 10^5/cm^2) were seeded on the dermis to obtain EHS. These were grown under submerged conditions in DMEM-supplemented culture medium lacking hydrocortisone. To generate an intact stratum corneum, the EHS was grown for 5 additional days at the air-medium interface.

UV Irradiation. EHS, keratinocytes, and fibroblasts for in vitro UV exposure as well as naked DNA for in vitro exposure were irradiated on ice. Naked DNA was diluted in physiological buffered solution consisting of 150 mM KCl, 10 mM NaCl, 1 mM EDTA, and 10 mM Tris-HCl (pH 7.4) at a concentration of 65 μg/ml. For UV irradiation, one UVB component and one UV-A component were used for EHS, keratinocytes, and fibroblasts to model the UVB spectrum as it would be absorbed by human skin in vivo.

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The abbreviations used are: CPD, cyclobutane pyrimidine dimer; EHS, engineered human skin; SSL, simulated sunlight; DMEH, DMEM-Ham’s; LMPCR, ligation-mediated PCR.
exposure of EHS, the epidermis was separated from the dermis, and then the stratum corneum was peeled off using a fine tweezer. The keratinocytes were isolated, and DNA was extracted as described (11). The global CPD frequency was determined with 1.5% alkaline agarose gels after T4 endonuclease V cleavage of the CPD, as described previously in detail (12).

LMPCR. Details of the LMPCR protocol have already been published (11, 13). Briefly, monolayer cells or EHS were irradiated with 254-nm UVC, UVB, and SSL. The genomic DNA was isolated immediately and digested with T4 endonuclease V to incise DNA at CPD, and the resulting 5’-pyrimidine overhang was removed by photoreactivation using Escherichia coli photolyase to generate ligatable ends (13, 14). A gene-specific oligonucleotide was annealed downstream of the break site, and a set of genomic cleavage products was extended using Sequenase. An asymmetric double-stranded linker was then ligated to the phosphate groups at the fragment termini, providing a common sequence on the 5’ end of all fragments. The longer oligonucleotide of this same linker, in conjunction with another gene-specific primer, was then used in a PCR to amplify the cleavage products of interest. These products were visualized alongside a Maxam and Gilbert sequencing ladder, and the intensities of the bands produced in UV-irradiated DNA are proportional to the amount of damage at the corresponding sites on the sequencing ladder. Primers specific for the human p53 gene were selected and used as described previously (4). Primer extension, which was initiated with 0.96–1.6 μg of DNA, and ligation of the linker, PCR, gel electrophoresis, electoblotting transfer, probe preparation, and hybridization were carried out as reported previously (11, 13). Each experimental condition was assayed in triplicate. A screening sequencing gel was run using a portion of the DNA to ensure that there was no significant variation between samples. The three samples were then pooled on a combined gel, and the autoradiogram was analyzed using a PhosphoImager (15).

Results and Discussion

For investigations of sunlight-induced DNA damage and mutation, the use of either pure cutaneous monolayer cell cultures (i.e., mainly keratinocytes or fibroblasts), animal models, or human skin biopsies presents major limitations: (a) pure monolayer cell cultures are clearly...
Fig. 3. Distribution of CPD along p53 exon 7 on the nontranscribed strand (A) and along p53 exon 5 on the transcribed strand (B). As a reference, a small portion of the Maxam-Gilbert-derived sequence is shown on the right. Codons 247–248 (A) and codons 154–153, 171–170, and 186–185 (B) are indicated. C°°, a methylated cytosine. A, Lanes 1–4, LMPCR of DNA treated with standard Maxam-Gilbert cleavage reactions; Lanes 5, 9, and 11, LMPCR of 254-nm UV-, UVB-, and SSL-irradiated purified DNA (†* in vitro) followed by T4 endonuclease V/photolyase digestion. Lanes 6, 10, and 11; LMPCR of 254-nm UV-, UVB- and SSL-irradiated fibroblasts (K*°) followed by T4 endonuclease V/photolyase digestion. Lanes 13 and 18, LMPCR of 254-nm UVB- and SSL-irradiated human skin (HS*) followed by T4 endonuclease V/photolyase digestion. Lane 19, LMPCR of unirradiated DNA followed by T4 endonuclease V/photolyase digestion. Keratinocytes K1 and K2 represent two sets of Petri dishes irradiated and harvested at different times, showing that the results are reproducible. B, Lanes 5–9, LMPCR of 254-nm UV-irradiated purified DNA, fibroblasts, keratinocytes, and human skin followed by T4 endonuclease V/photolyase digestion. Because the 254-nm UV-induced CPD formation is much less frequent in human skin than in fibroblasts and keratinocytes, there is much more DNA in Lane 9 to achieve a comparable band intensity.

very different from multilayered human skin in situ; (b) species differences often render extrapolations to the human situation extremely difficult; and (c) the use of human volunteers is unreliable and entails ethical problems. The use of EHS significantly mitigates each of these limitations, thereby constituting a critical alternative model system. Indeed, as illustrated in Fig. 1 and confirmed by previous histological studies and electron microscopy (16, 17), EHS presents dermal and epidermal structures comparable to normal skin. The reconstructed dermis is composed of well-oriented fibroblasts within an extracellular matrix containing both type I and type III collagens. It is particularly pertinent in terms of this study to note that EHS presents a structurally intact epidermis manifesting normal keratin distribution (18, 19), together with well-organized desmosomes, stratum corneum, stratum granulosum, stratum spinosum, and stratum germinativum (basal layer; Refs. 8 and 16). The stratum corneum in EHS, in a similar manner to normal skin, consists of many layers of flat, anucleate cells characterized by thickened plasma membranes, with the cytoplasm replaced by keratin. The processes by which cells were joined in the spiny layer are no longer visible, and the lifeless cells are closely packed together without any apparent interstices. Therefore, the stratum corneum and multilayered organization of the epidermal cells in EHS, as for human skin in situ, would clearly be expected to attenuate UV penetration to the deepest layers of the stratum spinosum and stratum germinativum, thereby protecting these tissues against the formation of carcinogenic UV-induced photoproducts.

Preceding studies have examined CPD formation either in cultured cell lines or in human or murine skin, following exposure to particular UV wavelengths (i.e., usually either 254-nm UVC or polychromatic UVB). To our knowledge, no investigations have rigorously examined CPD formation following exposure to different wavelengths in diverse model systems, nor has EHS been used for such purposes. Such comparative analyses are necessary to directly evaluate the capacity of the upper epidermal layers to attenuate specific UV wavelengths. We, therefore, quantified the initial formation of CPD in monolayer cells (cutaneous fibroblasts and keratinocytes) as well as in EHS, following exposure to 254-nm UVC, UVB, and SSL. The measurement of CPD provides not only a highly relevant biological end point with respect to photocarcinogenesis but also a valid indicator of UVB penetration through skin, because CPDs are formed almost exclusively via direct absorption of UVB photons by DNA.

Following irradiation of purified DNA, monolayer cells, or EHS with 254-nm UVC, UVB, or SSL, the DNA was immediately extracted and cleaved with the (CPD-specific) enzyme T4 endonuclease V, to convert virtually all of the CPD to single-strand breaks. The average break frequency was then directly correlated with the global CPD frequency within genomic DNA via gel-mobility shift analysis on denaturing agarose gels, as described previously (12, 20). Irradi-
ation of purified DNA (in vitro) with 1 kJ/m² 254-nm UV, 50 kJ/m² UVB, and 5000 kJ/m² SSL each yielded equivalent CPD levels, i.e., 64–68 adducts per 10 kb (Fig. 2, A, Lanes 12–14, and B, Lanes 12–14). When pure fibroblast or keratinocyte monolayers were irradiated under the same conditions, between 35 and 68 CPDs per 10 kb were initially formed for each treatment (Fig. 2, A, Lanes 15–20, and B, Lanes 15–17). A reduced amount of CPD (only 8 adducts per 10 kb) was generated when EHS was exposed to 1 kJ/m² 254-nm UV. This result was fully expected and demonstrates that the stratum corneum and multilayered organization of the EHS protect against 254-nm UV-induced CPD (Fig. 2B, Lane 18). However, fully 60–65 CPDs per 10 kb were generated when EHS was exposed to either 50 kJ/m² of UVB or 5000 kJ/m² of SSL (Fig. 2B, Lanes 19 and 20). Thus, in striking contrast to the situation for 254-nm UVC, CPD induction levels were virtually identical for EHS, keratinocytes, and fibroblasts, following irradiation with either UVB or SSL. It, therefore, appears that the stratum corneum and multilayered organization of human skin offer virtually no protection against penetration of environmentally relevant UVB and SSL to the cancer-prone basal layer of the epidermis.

In another approach, CPD formation was quantified at nucleotide resolution along exons 5–8 of the human p53 gene using the LMPCR. The LMPCR protocol, which has been described in detail elsewhere (Refs. 11 and 13; see “Materials and Methods” for overview), can be used to investigate any DNA adduct (including CPDs) that can be converted to a ligatable strand break following enzymatic or chemical cleavage. Compared to the situation for 254-nm UV and in general agreement with previous studies (15, 21), UVB- and SSL-irradiated cells manifested enhanced CPD formation at the majority of cytosine homodimers along the p53 gene, with a corresponding relative reduction in damage levels at TT homodimers. This enhanced CPD formation was especially remarkable at methylated dipyrimidine sites, i.e., CCmG and TCmG. Taking these differences into account, the comparative LMPCR data presented here clearly demonstrate, in accordance with the global CPD measurements presented above, that overall levels of CPD within the p53 gene are significantly attenuated in EHS versus monolayer cells irradiated with 254-nm UVC but that there is virtually no such difference following treatment with either UVB or SSL (Figs. 3 and 4). It should also be mentioned that because fibroblasts, keratinocytes, and EHS bear diverse cellular properties, one

Fig. 4. Distribution of CPD along the transcribed strand of p53 exon 7 (A) and p53 exon 8 (B). As a reference, a small portion of the Maxam-Gilbert-derived sequence is shown on the right. Lanes 1–4, LMPCR of DNA treated with standard Maxam-Gilbert cleavage reactions; Lanes 5–7, LMPCR of UV-irradiated purified DNA (In Vitro) followed by T4 endonuclease V/photolyase digestion; Lanes 8–13, LMPCR of UV-irradiated fibroblasts (Fibro, Lanes 8–10) and keratinocytes (Kerato1, Lanes 11–13) followed by T4 endonuclease V/photolyase digestion; Lane 14, LMPCR of unirradiated DNA followed by T4 endonuclease V/photolyase digestion. Lanes C, 254-nm UV; Lanes B, UVB; Lanes SL, SSL; Cm, methylated cytosine. Boxed triplets, codons 245 and 248; arrows, mutational hotspots in codons 245 and 248 (A). Arrows, dipyrimidine-methylated sites at codons 283–282, 298–297, and 307–306 (B).
might expect differences in CPD distribution patterns at the DNA sequence level, i.e., on a site-by-site basis. It is, therefore, noteworthy that the initial CPD distribution patterns were very similar for keratinocytes, fibroblasts, and EHS under all irradiation conditions, demonstrating that specific cellular properties do not significantly affect the sequence specificity of CPD formation (Figs. 3 and 4).

Our demonstration that the upper layers of the epidermis in EHS provide virtually no protection against carcinogenic DNA damage induced by environmentally relevant sunlight may have fundamental implications for the prevention of cutaneous tumors. Indeed, whereas skin cancer is currently the most frequent neoplasia in Caucasian populations, the incidence of this disease can be vastly reduced through heightened awareness, among clinicians and the public alike, of the necessity to avoid direct solar UV exposure. The possibility that the upper epidermis represents an ineffective barrier to solar UVB penetration provides powerful reinforcement for the importance of regular sunscreen use, which may constitute a far more critical (and highly) effective first line of defense against the initiation of photocarcinogenesis than previously believed. Moreover, in the case of fair-skinned individuals endowed with only very low levels of melanin, sunscreens may indeed represent the only primary source of protection against the induction of DNA photoproducts in the skin cancer-prone basal layer of the epidermis.

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References

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