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Advances in Brief

Sunlight Induces Pyrimidine Dimers Preferentially at 5-Methylcytosine Bases[1]

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Abstract

The most prevalent DNA lesion induced by UV irradiation is the cyclobutane pyrimidine dimer (CPD), which forms at positions of neighboring pyrimidines. Here we show that the rare DNA base 5-methylcytosine is the preferred target for CPD formation when cells are irradiated with natural sunlight. We have mapped the distribution of CPDs formed in normal human keratinocytes along exons of the *p53* gene. Codons 196, 245, 248, and 282, which are mutational hot spots in skin cancers, are only weakly to moderately susceptible to formation of CPDs after irradiation with UVC (254 nm) or UVB (320 nm) light sources. However, when cells were exposed to natural sunlight, CPD formation was enhanced up to 15-fold at these codons due to the presence of 5-methylcytosine bases. These results suggest that CPDs containing 5-methylcytosine may play an important role in formation of sunlight-induced skin tumors and that methylation of CpG sequences, besides being involved in spontaneous mutagenesis processes, can also create preferential targets for environmental mutagens and carcinogens.

Introduction

More than 500,000 new cases of skin cancer are diagnosed each year in the United States. Exposure to solar radiation is a principal factor in the development of skin cancer (1, 2). Mutations in the *p53* tumor suppressor gene have been found in a large percentage of human skin malignancies (see, *e.g.*, Refs. 3–8) and in precursor lesions (9, 10), and even normal sun-exposed skin contains clonal patches of *p53*-mutated keratinocytes (11). The predominant base changes are C-to-T and CC-to-TT mutations at dipyrimidine sequences, two types of base alterations induced specifically by UV light in many experimental systems (12, 13). Of the various types of lesions formed in DNA after UV irradiation, the CPD[3] is considered the most mutagenic lesion based on its abundance, slow repair, and distinct mutagenicity (14).

In the *p53* gene of human skin cancers, many C-to-T transition mutations are within CpG sequences, suggesting that many of these mutations could have been UV induced or, alternatively, could have resulted from spontaneous deamination of 5-mC bases at CpGs (15). Our study demonstrates that by examining the distribution of CPDs along the *p53* gene, an important role of 5-mC in sunlight-associated mutagenesis can be defined.

Materials and Methods

Cell Culture and Irradiation. Normal human foreskin keratinocytes were grown in serum-free growth medium. Before UV irradiation, the cells were washed in PBS. The UVC light source was a 254-nm germicidal lamp. The UVB source was a Philips TL 20W/12RS lamp filtered through a clear polystyrene dish (peak emission, 320 nm; lower wavelength cutoff, 295–300 nm). Keratinocytes covered with a layer of 2 mm of PBS were sun-irradiated on ice for 2–3 h around noon on a cloudless June day in Los Angeles County, California. Nonirradiated cells served as negative controls.

DNA Isolation and Mapping of CPDs. DNA isolation, enzymatic cleavage at CPDs, and ligation-mediated PCR were done as described previously (16, 17). Oligonucleotide primer sets specific for sequences of the human *p53* gene (18, 19) were used to map CPDs in irradiated DNA and cells. The frequency of CPD appearance in the total genome was estimated by separation of T4 endonuclease V cleavage products on a 0.6% alkaline agarose gel.

Methylation and Irradiation of Plasmid DNA. A plasmid containing genomic sequences of the human *p53* gene encompassing exons 2–11 was methylated *in vitro* using the CpG-specific methylase *Sss*I (New England Biolabs, Beverly, MA). Control DNA was mock methylated in the absence of SAM. Completion of methylation was confirmed by digesting an aliquot of the reaction mixture with the methylation-sensitive restriction endonuclease *Hpa*II and by Maxam-Gilbert sequencing. Methylated and unmethylated DNAs were irradiated with UVC, UVB, or sunlight. CPDs were then mapped by T4 endonuclease V cleavage and ligation-mediated PCR as described (16, 17).

Results

The distribution of mutations along the *p53* gene of skin cancers is characterized by several mutational hot spots (Fig. 1). To determine whether this mutational spectrum is related to the distribution pattern of CPDs along the same sequences in UV-exposed cells, we have irradiated human keratinocytes with UVC or UVB light sources or with natural sunlight under conditions that produce similar lesion frequencies (Fig. 2). The irradiation conditions introduced approximately one CPD every 2–3 kb of DNA.

CPDs were then mapped in the *p53* gene by ligation-mediated PCR (16, 17). Fig. 3 shows an analysis of the upper strand of exon 6 and of upper and lower strands of exon 7. Exon 6 contains codon 196, which is a prominent mutational hot spot in skin cancers but not in internal tumors (Fig. 1; Ref. 20). There is a progressive enhancement of CPD formation from UVC to UVB and to natural sunlight at codons 196 and 213, both of which contain dipyrimidines within a CpG sequence (Fig. 3*A*). Other sequences along the same exon differ only slightly (less than a factor of 2) in their susceptibility to damage formation by the different UV sources. In fact, under conditions of solar radiation (Fig. 3*A*, *Lanes 9* and *10*), codon 196 becomes the most significantly damaged position along the entire exon. Of the 25 mutations observed at codon 248 in exon 7, 17 are C-to-T transitions on the upper (nontranscribed) strand within the sequence 5′-CCGG (codon 248 is underlined). Codon 248 does not form significant amounts of CPDs after 254 nm (UVC) irradiation on the upper strand (Fig. 3*B*; Ref. 18). However, this codon is approximately five times more susceptible to solar radiation damage compared to UVC (as quantitated by phosphor imaging). The same sequences on the opposite DNA strand were analyzed in Fig. 3*C*. In the available literature (Fig. 1), eight mutations can be ascribed to the dipyrimidine sequence at codon 248 of this strand, and seven are located at codon 245, which contains a dipyrimidine sequence only on the lower strand. The analysis shows that these two dipyrimidine sequences within the sequence context CpG are 5- to 15-fold more susceptible to CPD

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[3] The abbreviations used are: CPD, cyclobutane pyrimidine dimer; 5-mC, 5-methylcytosine; SAM, *S*-adenosylmethionine.

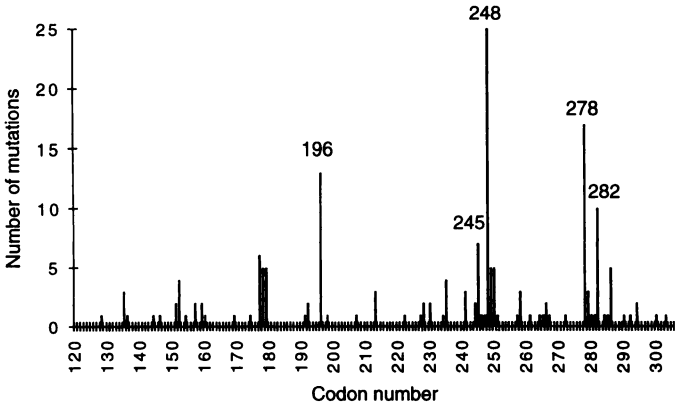

Fig. 1. Mutational spectrum of the human *p53* gene in skin cancer. The data include mutations from nonmelanoma skin cancers, precursor lesions, and normal sun-exposed skin. Numbers were obtained from an updated *p53* mutation database (20). Only mutations affecting dipyrimidine sequences were included (these are more than 90% of all mutations). The vast majority of these mutations are C-to-T transitions.

formation by sunlight compared to UVC or UVB in irradiated keratinocytes (Fig. 3*C*).

The apparent enhancement of solar radiation susceptibility occurs almost exclusively at CpG sequences. Methylation of these sequences, which is ubiquitous in human tissues including keratinocytes (19), may contribute to enhanced CPD formation after solar irradiation. To test this hypothesis, we have irradiated *p53* sequences present in plasmid DNA in an unmethylated form and compared the pattern to that obtained when the plasmid was methylated with the CpG-specific DNA methylase *Sss*I (Fig. 4, *A* and *B*). The results show that cytosine methylation clearly increases solar radiation susceptibility at CpG codons but does not enhance CPD formation after UVC treatment. Both methylated 5′-CCG and 5′-TCG sequences are prone to this effect. Interestingly, there is also an enhancement in irradiated plasmid DNA by UVB and sunlight (Fig. 4*B*, *Lanes 6, 7, 9,* and *10;* *) near codon 260 that is independent of the CpG methylation status controlled by *Sss*I methylase. The affected sequence, 5′-CCTGG, is a

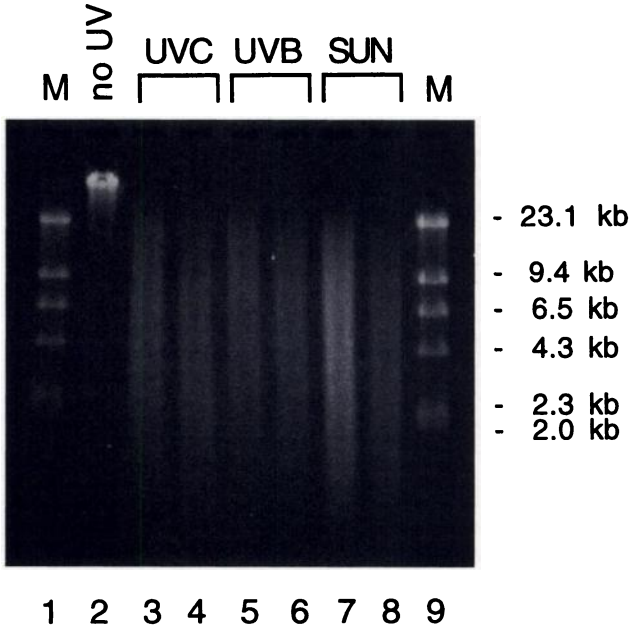

Fig. 2. Global CPD frequency in DNA from cells irradiated with UVC, UVB, or sunlight (*SUN*). Keratinocytes were irradiated with doses of 45 and 60 J/m^2 UVC (*Lanes 3* and *4*, respectively), 1500 and 2000 J/m^2 UVB (*Lanes 5* and *6*), or for 2 and 3 h with sunlight (*Lanes 7* and *8*). *Lane 2*, unirradiated DNA. DNA was isolated, digested with T4 endonuclease V, and separated on a 0.6% alkaline agarose gel. *M*, molecular weight marker; λ DNA digested with *Hin*dIII.

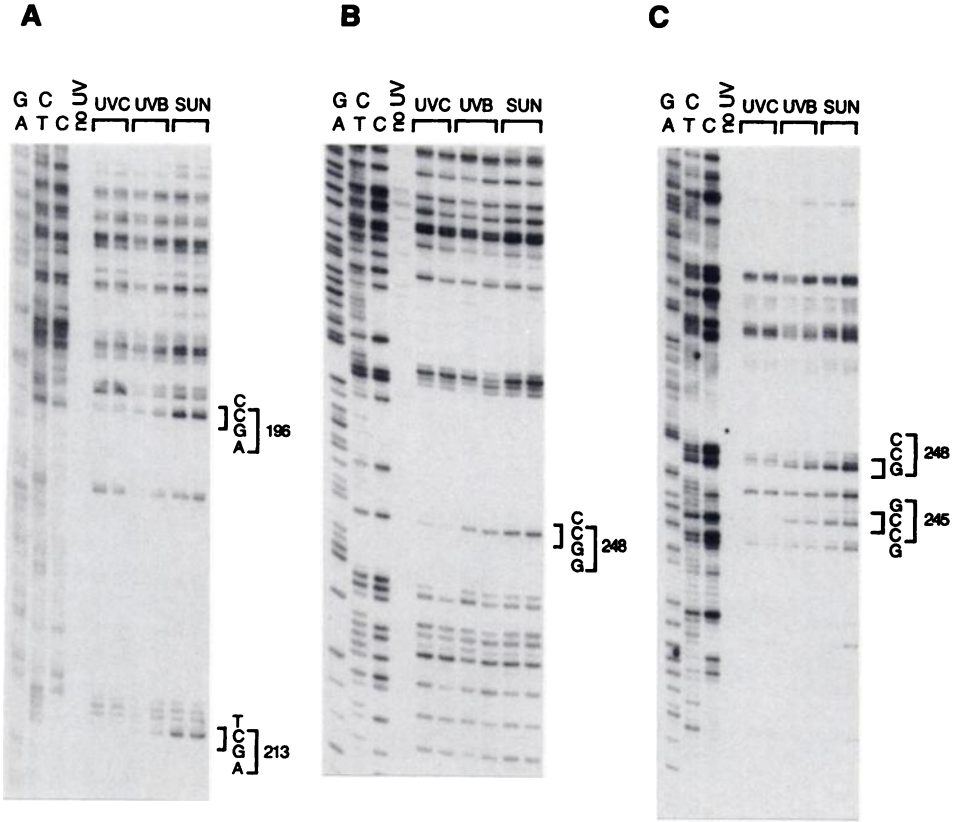

Fig. 3. Mapping of CPDs along exons of the *p53* gene. *A*, exon 6, upper strand; *B*, exon 7, upper strand; *C*, exon 7, lower strand. *Lanes 1–3*, Maxam-Gilbert sequencing control lanes. *Lane 4*, unirradiated DNA. Human keratinocytes were irradiated with UVC (45 and 60 J/m^2; *Lanes 5* and *6*, respectively), UVB (1500 and 2000 J/m^2; *Lanes 7* and *8*), or natural sunlight (2 and 3 h; *Lanes 9* and *10*). DNA was isolated immediately after irradiation and cleaved at CPD sites. CPDs were detected by ligation-mediated PCR using *p53*-specific primers. CPDs are enhanced after solar irradiation at the indicated codons, which include skin cancer mutational hot spot codons 196, 245, and 248.

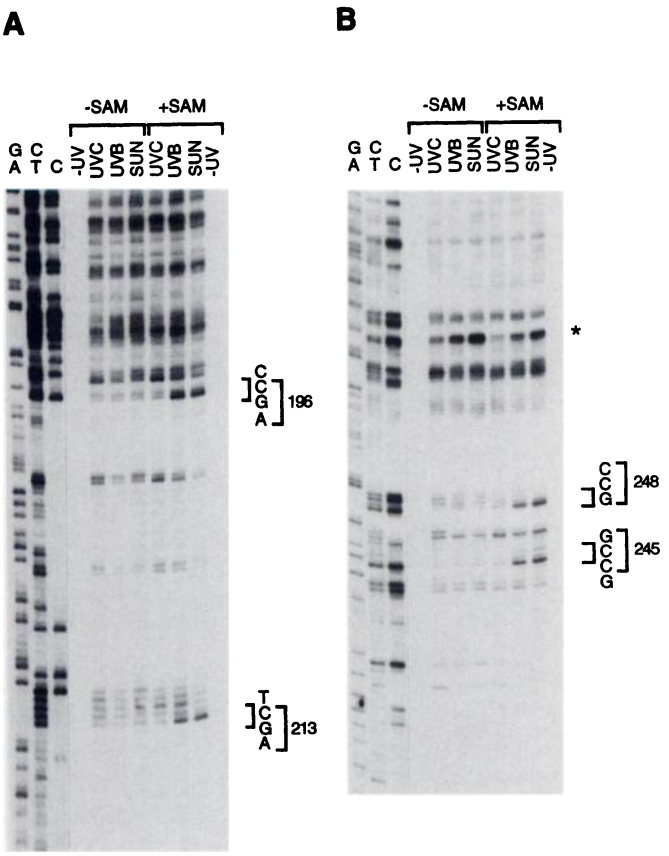

Fig. 4. Mechanism of enhanced CPD formation by solar radiation. CPDs were mapped along the nontranscribed (upper) strand of exon 6 (*A*) and the transcribed (lower) strand of exon 7 (*B*) of the *p53* gene. The effect of cytosine methylation on formation of CPDs after UVC, UVB, and solar irradiation was analyzed. Plasmid DNA containing *p53* sequences was either mock methylated (−SAM) and was irradiated with UVC (45 J/m^2), UVB (1500 J/m^2), or sunlight (1.5 h), or was methylated at all CpG sites with the DNA methylase *SssI* (+SAM) and was subsequently UV irradiated under the same conditions. Methylation leads to an enhancement of CPD frequency at methylated CpG sequences (codons 196, 213, 245, and 248, indicated by *brackets*) by solar and UVB irradiation. Methylation of a *dcm* site (*) in plasmid DNA grown in *E. coli* also leads to increased CPD formation by UVB and sunlight, which is independent of the CpG methylation status.

dcm methylation site. Because the plasmid was propagated in *Escherichia coli*, these sites are methylated to form 5′-CmCTGG. Presence of 5-mC at this sequence enhances CPD formation by solar radiation, and a shorter exposure of the autoradiograms showed that both the 5′-CmC and 5′-mCT dipyrimidines were affected.

One other site of frequent mutation in skin cancer is codon 278 (Fig. 1), which lacks a CpG sequence. This codon does not show an enhancement of CPD formation by solar radiation in irradiated keratinocytes (Fig. 5). However, codon 290, which is within a CpG sequence, shows a dramatic enhancement of CPDs formed after solar radiation (Fig. 5*A*), and this increase is methylation dependent (Fig. 5*B*). Codon 282 shows a higher susceptibility to sunlight and also contains a CpG sequence. This sequence is one of the more commonly mutated sites in skin cancers (Fig. 1).

Discussion

It was realized previously that the sequence-specific distribution of UV-induced DNA photoproducts can be wavelength dependent. A 1.2- to 2.1-fold enhancement of CPD formation was observed at the dipyrimidines 5′-TC, 5′-CT, and 5′-CC with UVB irradiation when compared to UVC (21). Here, we report that methylation of cytosines has a much more dramatic effect on CPD enhancement in the solar UV range. The λmax of 5-mC *versus* cytosine is red-shifted by about 6 nm. At neutral pH, the λmax of 5-mC is 273.5 nm, and the λmax of cytosine is 267 nm (22). At pH 5, these numbers are 277 and 269 nm, and at pH 12, they are 277.5 and 272 nm for 5-mC and cytosine, respectively. The red shift results in a pH- and wavelength-dependent 5–10-fold higher extinction coefficient for 5-mC *versus* cytosine at wavelengths between 300 and 315 nm (22). This part of the solar spectrum represents sunlight that reaches the earth's surface and is absorbed by DNA (23). In addition, 5-methyl-dCMP has a significantly lower excited singlet state energy than TMP and dCMP (24), and therefore, 5-methyl-dCMP could serve as a singlet energy trap in DNA.

Because methylated cytosines occur only at CpG dinucleotides in mammalian DNA, this might explain why many of the skin cancer mutational hot spots in the *p53* gene are at CpGs. A high frequency of photoproduct formation at these codons could drive mutagenesis. It is predicted that methylated CpG sequences in other genes will also be preferential targets for sunlight-induced mutagenesis.

Not all sequences that can form CPDs at high frequency are found as mutational hot spots in skin cancers (*e.g.*, codon 290; Fig. 5), and *vice versa*, codons that contain many mutations, such as codon 248 on the upper strand (Fig. 3*B*) and codon 278 (Fig. 5), are not the strongest targets for CPD formation. Thus, additional pathways will likely play

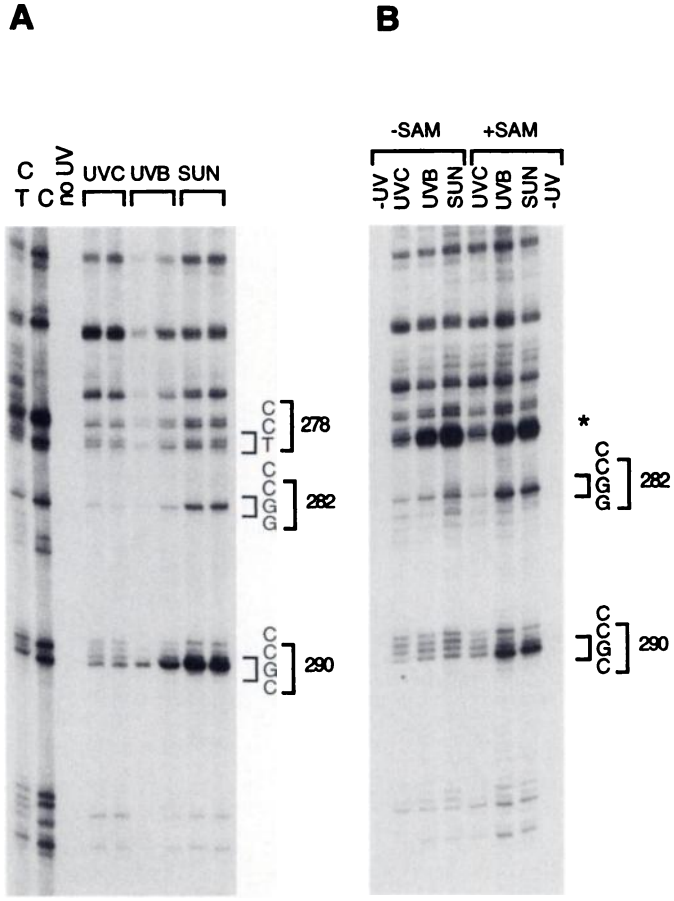

Fig. 5. Mapping of CPDs along the nontranscribed DNA strand of exon 8 of the *p53* gene. *A*, human keratinocytes were irradiated with UVC (45 and 60 J/m^2; *Lanes 4* and *5*, respectively), UVB (1500 and 2000 J/m^2; *Lanes 6* and *7*), or natural sunlight (2 and 3 h; *Lanes 8* and *9*). DNA was isolated, cleaved at CPD sites, and subjected to ligation-mediated PCR using *p53*-specific primers. CPDs are enhanced after solar irradiation at codons 282 and 290, indicated by *brackets*. *B*, effect of cytosine methylation on formation of CPDs after UVC, UVB, and solar irradiation. See Fig. 4 legend for details. Methylation leads to an enhancement of CPD frequency at methylated CpG sequences (codons 282 and 290, indicated by *brackets*) by solar and UVB irradiation. Note that codon 278 is contained within a *dcm* methylation site and shows enhanced CPD formation by solar radiation in plasmid DNA only (*).

a role in the UV mutagenesis process leading to *p53* mutations. A significant contributing factor is probably that CPDs at codons 177, 196, 245, 248, and 278 are repaired inefficiently, as shown previously (25). Although considered less likely, the major mutagenic lesion at some sequences may be the (6-4) photoproduct (14) or even a minor photoproduct. Mutations may or may not impart a selective advantage during the cell transformation process. For example, codon 290, which is a prominent site of CPD formation by sunlight in exon 8 (Fig. 5), is represented only nine times in the >5000-entry *p53* mutation database of all tumors (20). Of these nine point mutations, three are silent substitutions, suggesting that mutations at codon 290 may not be strongly tumorigenic. An additional possibility is that most UV-induced transition mutations at dipyrimidines containing cytosine may result from correct DNA polymerase bypass of CPDs containing deaminated cytosine or 5-mC (26–28). Deamination of C in T-C or C-C dimers leads to formation of T-U or U-U dimers, respectively, and deamination of 5-mC will result in T-containing dimers. Adenines are incorporated with high specificity during bypass of site-specific T-T, T-U, or U-U dimers (27, 29). Deamination of 5-mC in CPDs may be generally more efficient than that of cytosine, which, together with the increased induction of such CPDs in the solar UV range, could contribute significantly to enhanced mutagenesis of dipyrimidines located within the methylated CpG sequences of codons 196, 245, 248, and 282.

We have shown previously that methylated CpG dinucleotides represent selective modification sites for a chemical carcinogen of the polycyclic aromatic hydrocarbon class (30, 31). The results presented here provide an additional example showing that methylated CpGs, besides being involved in spontaneous mutagenesis, can also form preferential targets for exogenous mutagens and carcinogens.

Note Added in Proof

Drouin and Therrien report increased formation of CPDs at CpG sequences in the UVB range (R. Drouin and J-P. Therrien, UVB-induced CPD frequency correlates with skin cancer mutational hotspots in p53. Photochem. Photobiol., in press, 1997).

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