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Influence of cytosine methylation on ultraviolet-induced cyclobutane pyrimidine dimer formation in genomic DNA

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ABSTRACT

The ultraviolet (UV) component of sunlight is the main cause of skin cancer. More than 50% of all non-melanoma skin cancers and >90% of squamous cell carcinomas in the US carry a sunlight-induced mutation in the *p53* tumor suppressor gene. These mutations have a strong tendency to occur at methylated cytosines. Ligation-mediated PCR (LMPCR) was used to compare at nucleotide resolution DNA photoproduct formation at dipyrimidine sites either containing or lacking a methylated cytosine. For this purpose, we exploited the fact that the X chromosome is methylated in females only on the inactive X chromosome, and that the FMR1 (fragile-X mental retardation 1) gene is methylated only in fragile-X syndrome male patients. Purified genomic DNA was irradiated with UVC (254 nm), UVB (290–320 nm) or monochromatic UVB (302 and 313 nm) to determine the effect of different wavelengths on cyclobutane pyrimidine dimer (CPD) formation along the X-linked *PGK1* (phosphoglycerate kinase 1) and *FMR1* genes. We show that constitutive methylation of cytosine increases the frequency of UVB-induced CPD formation by 1.7-fold, confirming that methylation *per se* is influencing the probability of damage formation. This was true for both UVB sources used, either broadband or monochromatic, but not for UVC. Our data prove unequivocally that following UVB exposure methylated cytosines are significantly more susceptible to CPD formation compared with unmethylated cytosines.

1. Introduction

Exposure to the ultraviolet (UV) component of sunlight is the preeminent risk factor in skin cancer development [1,2]. Cyclobutane pyrimidine dimers (CPDs) are the primary promutagenic DNA adducts generated by all UV wavelengths, i.e., UVC (200–280 nm), UVB (280–320 nm) and UVA (320–400 nm) [3–5]. UV also induces (6-4) pyrimidine-pyrimidone photoproducts (6-4PPs) and oxidative DNA damage to a lesser extent. Although 6-4PPs were found to be mutagenic in *E. coli* [6], CPDs are considered the most important for skin carcinogenesis based on their relative abundance (approximately 85% of DNA adducts after UV), relatively slow removal by nucleotide excision repair, and known high mutagenic potential in human cells [7,8].

The *p53* tumor suppressor gene is mutated in more than 50% of all cancers and in approximately 90% of squamous cell carcinomas of the skin [9,10]. The vast majority of *p53* mutations in non-melanoma skin cancers are C→T and CC→TT tandem double transition mutations, considered the "mutational signature" of UV exposure [11]. Such signature mutations preferentially occur at 5′YCG sequences (i.e. 5′TCG or 5′CCG). In fact, even though 5′YCG represents only 3% of all trinucleotides in *p53*, more than 30% of all tumor-derived mutations occur at these sites [12]. Of the 8 most common hotspots for *p53* mutations in skin cancer, 6 occur at the cytosine of a 5′YCG sequence [13,14]. In vertebrates, the cytosine in a 5′CG sequence is a target for DNA methylation at position 5 of the pyrimidine ring (5^{m}CG) [15]. In the coding region of exons 5–8 of the human *p53* gene, 42 5′CG sites are fully methylated [12]. In the case of internal cancers, it is believed that a high frequency of spontaneous deamination of 5-methylcytosine (5^{m}C) to thymine at 5′CG sites in *p53* engenders the observed preponderance of C to T transitions at these sites [9,16].

A 5^{m}CG preference for UV-induced mutations in the *p53* gene is not observed after UVC exposure [17,18], but is clearly observed after UVB [17,18]. Moreover, the 5^{m}CG sensitivity to UVB seems

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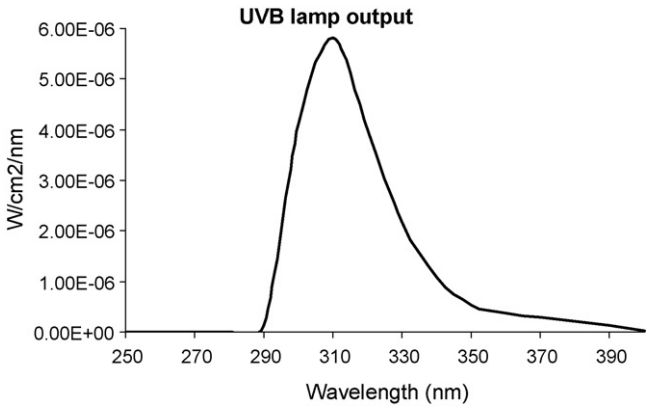

Fig. 1. Emission spectrum of the UVB lamp (FS20/T12/UVB/BP) after filtering through a Kodacel TA-407 clear 0.015 in. filter. Measurements were made using an International Light double monochromator spectroradiometer (IL1700/760D/790).

to increase with the wavelength. In a mouse model, UV-specific tumor mutations in *p53* occurred at 5^{m}CG sites at a frequency of 33% following irradiation with UVB (290–320 nm), 43% with broad spectrum simulated sunlight (SSL, >300 nm), and 66% with UVA (320–400 nm) [19–21].

What might explain this wavelength-dependent specificity for C → T mutations at 5^{m}CG sites? CPDs are efficiently bypassed in a relatively error-free manner by DNA polymerase η (encoded by *XPV* gene). When polymerase η bypasses a 5′TT, 5′TU or 5′UU dimer (T or U generated by deamination of 5-methylcytosine or cytosine, respectively), adenine is incorporated with high preference across from T or U [22–24], thereby generating a C → T mutation. The half-life for cytosine deamination in dsDNA is ∼25,000 years [25–27] and ∼2000 years when methylated [28]. However, when cytosine is covalently bound to another pyrimidine to form a CPD, the deamination half-life is reduced to 2–100 h [25,27,29–31]. When the same cytosine is methylated in a CPD, the deamination is further accelerated [32]. As a result, when implicated in a CPD, cytosine methylation increases the probability of generating a mutation. Moreover, (i) CPD repair in Chinese hamster ovary cells is slower when the DNA is methylated [33,34] and (ii) CPD formation is actually enhanced by the methylation of cytosines. Indeed, some *p53* mutational hotspots found in non-melanoma skin cancers at 5′YCG sites are as much as 15-fold more susceptible to CPD formation after exposure to sunlight (>295 nm) [13] or UVB [17]. On synthetic residues, Mitchell observed a 2-fold increase in UVB-induction of CPD at a methylated cytosine when compared with the cytosine at the same position but unmethylated [35]. On the other hand cytosine methylation did not enhance CPD formation by UVC [36]. In conclusion, the methylated cytosines in dipyrimidine sites are the nucleotide residues with the highest mutation rate upon UVB and SSL irradiation. One hypothesis frequently proposed to explain the results is the different UV absorbance spectra for C and 5^{m}C, which manifest a maximum at 267 and 273.5 nm, respectively [37,38].

In the studies cited above, the efficiency of different UV wavelengths for inducing CPD at a given 5′Y^{m}CG site was compared. However, comparisons of the same 5′YCG site when methylated versus unmethylated using endogenous genomic sequences has not yet been performed. We therefore analyzed the influence of cytosine methylation on UV-induced CPD formation by comparing the same 5′YCG methylated or not in genomic DNA. To do so, we exploited the natural methylation-related inactivation of the X chromosome. In female mammalian cells, one of the two X chromosomes becomes genetically silent during early embryogenesis. The inactivation of one of the X chromosomes in females is a methylation-related gene-silencing mechanism that affects almost all genes of the X chromosome (reviewed in [39]). *PGK1* (phosphoglycerate kinase 1) and *FMR1* (fragile-X mental retardation 1) are subject to strong transcriptional silencing by methylation-related X inactivation [40]. The *PGK1* 5′CG-rich island is unmethylated in the active X chromosome [41] and methylated at 119 of 121 5′CG dinucleotides in the inactivated X [42,43]. Female X-inactivation is not the only condition in which *FMR1* is methylated at 5′CG. In fragile-X syndrome male patients, the *FMR1* gene is mutated resulting in the hypermethylation of the gene, as in the inactivated X chromosome in female [44]. In the present study, we used ligation-mediated PCR (LMPCR) to analyze the efficiency of different UV wavelengths, i.e., UVC (254 nm) and UVB (290–320 nm, 303 and 313 nm) to generate CPD at 5′YCG sites that were methylated or not in the endogenous *PGK1* and *FMR1* genes. As such, we were able to unequivocally determine the effect of DNA methylation on CPD formation.

2. Materials and methods

2.1. Cell culture, DNA extraction and UV irradiation

DNA was harvested from human diploid fibroblasts of a male, a female, or a male with the fragile-X syndrome (mutated on the *FMR1* gene). For monochromatic irradiations, DNA was extracted from female human diploid lymphoblasts. DNA extraction from fibroblasts and lymphoblasts was performed as previously published [45]. Prior to irradiation, purified DNA was dissolved at a concentration of 60 μg/mL in irradiation buffer (150 mM KCl, 10 mM NaCl, 1 mM EDTA, and 10 mM Tris–HCl pH 7.4). The UVB (290–320 nm) source consisted of two fluorescent tubes (FS20/T12/UVB/BP, Philips, city) delivering a dose rate of 7.45 J/(m^2 s), which was filtered through a sheet of cellulose acetate to eliminate wavelengths below 290 nm (Kodacel TA-407 clear 0.015 in.; Eastman-Kodak Co., Rochester, NY). The emission spectrum of the UVB lamp is depicted in Fig. 1. A Philips G15T8 TUV 15W germicidal lamp was used to irradiate cells with 254 nm UV at a fluency of 6.25 J/(m^2 s). Doses for UVB and UVC irradiation (10,000 and 200 J/m^2, respectively) were chosen to give approximately 1 CPD per kb of genomic DNA [46]. For the monochromatic irradiations (GM 252, Spectral Energy Co. Washingtonville, NY), 302 and 313 nm were used at doses of 12,000 and 75,000 J/m^2, respectively. Doses were chosen to give similar CPD frequency as of polychromatic UVB and UVC.

2.2. Hpa II digestion of female DNA

In females, the active X chromosome is undermethylated whereas the inactive one is hypermethylated. In males, there is only one X chromosome and it is undermethylated. In this study, the goal was to use the hypermethylated inactive X chromosome from the female to compare it with the undermethylated X chromosome from the male. To do so, we had to eliminate the active X chromosome in the female DNA. Hpa II, a restriction enzyme sensitive to methylation, cleaves specifically at an unmethylated 5′CCGG sites allowing us to digest the active X chromosome in females leaving the inactive one for LMPCR analysis. This approach has been successfully used previously [42,47–49]. Ten units of Hpa II restriction enzyme (New England BioLabs) were used to cleave 10 μg of genomic DNA incubated 1 h at 37 °C.

2.3. Ligation-mediated PCR (LMPCR)

The LMPCR protocol has been described previously in detail [45]. Briefly, irradiated genomic DNA was extracted and digested with T4 endonuclease V to incise the DNA at CPD sites (including 5′YU dimers [50]). The resulting 5′-pyrimidine overhangs were then removed by photoreactivation using *E. coli* CPD photolyase, in order to generate ligatable ends. A gene-specific oligonucleotide was annealed downstream of the break site, and the set of genomic cleavage products was extended using cloned *Pfu exo-* DNA polymerase (Strategene, LaJolla, CA). The Hpa II digestion of the female DNA insured that only the molecules from the inactivated X chromosome could undergo this primer extension. An asymmetric double-stranded linker was then ligated to the phosphate groups at the fragment 5′ termini, providing a common sequence on the 5′-end of all fragments. A linker-specific primer, in conjunction with another gene-specific primer, was used in a PCR reaction to amplify the cleavage products of interest. These products were subjected to electrophoresis on 8% polyacrylamide sequencing gels alongside a Maxam and Gilbert sequencing ladder, transferred to nylon membranes, hybridized to a ^{32}P-labeled gene-specific probe, and visualized by autoradiography. Each experimental condition was assayed in duplicate. A screening sequencing gel was initially run using a portion of the DNA to ensure that there was no significant variation between samples. The two samples were then pooled on a combined gel, and the resulting autoradiogram analyzed using a Fuji BAS 1000 phosphorimager (Fuji Medical Systems, Stanford, CT, USA). Each band represents a nucleotide position where a break was induced by CPD cleavage, and the intensity of the band reflects the number of DNA molecules with ligatable ends at that position. Therefore, it is possible to make a quantitative correlation between the band intensity and the CPD formation at each dipyrimidine site.