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QUANTITATION AND VISUALIZATION OF ULTRAVIOLET-INDUCED DNA DAMAGE USING SPECIFIC ANTIBODIES

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Abstract : The major types of DNA damage induced by sunlight in the skin are DNA photoproducts, such as cyclobutane pyrimidine dimers (CPDs), (6-4)photoproducts (6-4PPs) and Dewar isomers of 6-4PPs. A sensitive method for quantitating and visualizing each type of DNA photoproduct induced by biologically relevant doses of UV or sunlight is essential to characterize DNA photoproducts and their biological effects. We have established monoclonal antibodies specific for CPDs, 6-4PPs or Dewar isomers. Those antibodies allow one to quantitate photoproducts in DNA purified from cultured cells or from the skin epidermis using an enzyme-linked immunosorbent assay. One can also use those specific antibodies with in situ laser cytometry to visualize and measure DNA photoproducts in cultured cells or in the skin, using indirect immunofluorescence and a laser-scanning confocal microscope. This latter method allows us to reconstruct three-dimensional images of nuclei containing DNA photoproducts, and to simultaneously examine DNA photoproducts and histology in multilayered epidermis. Using those techniques, one can determine the induction and repair of these three distinct types of DNA photoproducts in cultured cells and in the skin exposed to sublethal or suberythematous doses of UV or solar simulated radiation. As examples of the utility of these techniques and antibodies, we describe the DNA repair kinetics following irradiation of human cell nuclei and the photoprotective effect of melanin against DNA photoproducts in cultured pigmented cells and in human epidermis.

Key words : Cyclobutane pyrimidine dimer, (6-4)Photoproduct, Monoclonal antibody, ELISA, Immunofluorescence

INTRODUCTION

Sunlight generates various types of DNA damage such as DNA photoproducts and oxidative DNA damage[1,2]. More than 90% of the damage elicited by sunlight in the DNA of cultured cells are photoproducts that are induced exclusively by an ultraviolet (UV)-B component of sunlight[3]. DNA photoproducts are formed at dipyrimidine sites, ~75% of which are cyclobutane pyrimidine dimers (CPDs), the remaining ~25% being (6-4) photoproducts (6-4PPs) and Dewar isomers of 6-4PPs (Fig. 1). The major skin cancer threat

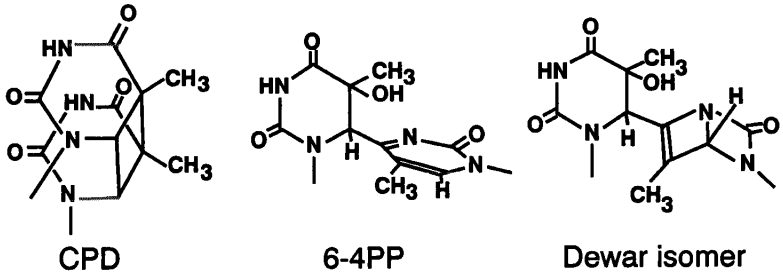

Fig. 1. DNA photoproducts. The UV-B component of sunlight produces two major types of DNA damage, cyclobutane pyrimidine dimers (CPD) and (6-4)photoproducts (6-4PP). Fig. 1 shows their chemical structures formed in thymine-thymine sequence. The CPD and the 6-4PP have a cyclobutane ring and a 6-4 covalent bond between adjacent pyrimidines, respectively. Both types of lesions lead to a considerable distortion of the phosphodiester backbone of DNA. A 6-4PP can be converted to a Dewar isomer by irradiation with 280 to 360 nm-UV light (with a peak at 320 nm).

comes from exposure to UV-B (contribution to skin cancer risk, >75%)[4]. The development of skin cancer is a multi-step process that can be initiated when these DNA photoproducts are not properly repaired by the nucleotide excision repair (NER) pathway[4,5]. The majority of mutations in the p53 gene in non-melanoma skin cancer on sun-exposed areas occur at dipyrimidine sites[6]. Mutations are also detected at dipyrimidine sites of the p16 gene in melanoma cells[7]. DNA photoproducts initiate immunosuppression[8], and thus may also stimulate later steps of skin cancer development.

Several methods are available for determining the induction and/or repair of DNA photoproducts, including chromatographic[9-12], enzymatic[13,14], agarose gel electrophoresis[3,15], plasmid host cell reactivation[16] and immunological[17-12] methods. The immunological method has several advantages over the others. For example, the induction and repair of DNA photoproducts can be determined in cultured cells or in the skin exposed to biologically relevant, sublethal or suberythematous, doses of UV[21-27]. Another advantage is that one can locate DNA lesions within an individual nucleus under a microscope[21,24,28-31]. Since Levine et al. first raised an antiserum against UV-irradiated single stranded DNA and developed an immunological method for measuring DNA photoproducts using complement fixation in 1966[17], a number of other antisera have been prepared in many laboratories for DNA photoproduct measurement with some modifications[18-31]. A polyvalent antiserum however, may contain a heterogeneous population of antibodies that recognize various kinds of DNA damage[25,32,33]. An immunological assay is an indirect method in which selective binding of an antibody to the corresponding antigen is essential and monoclonal antibodies are the most specific probes. We thus raised monoclonal antibodies against each type of DNA damage, CPDs, 6-4PPs or Dewar isomers[34-38]. We have also established an enzyme-linked immunosorbent assay (ELISA) for reliable and sensitive quantitation of these photoproducts[37,38]. This ELISA technique can be used for cultured cells and for intact tissue, such as the skin. Ford and Hanawalt[39] adapted these specific antibodies to an immunoblot assay. We have also used these specific antibodies with an in situ laser cytometric method to visualize and measure photolesions in cultured cells or in the skin, using indirect immunofluorescence and

a laser-scanning confocal microscope[1,40-43]. Potten *et al.* developed a similar method using immunohistochemistry and image analysis[44].

There is increasing interest in the roles of UV-induced DNA damage and/or its repair in pigment cell biology, including their role in melanogenesis (tanning)[45-47], photoprotection and/or photosensitization by melanin[43,48], melanomagenesis[49], etc. Techniques for the quantitation and visualization of UV-induced DNA damage as described in this review may contribute to a better understanding of DNA damage and its biological consequences. Although details of our techniques have already been reported, we will emphasize some practical points and recent improvements in our methods to assist pigment cell biologists who are interested in the photobiology of melanin.

MONOCLONAL ANTIBODIES

Nine distinct mouse monoclonal antibodies have been generated in our laboratory, each specific for CPDs, 6-4PPs or Dewar isomers (Table 1)[34-38]. We established TDM-2, TDM-3, 64M-2, 64M-3, 64M-4, and 64M-5 antibodies from BALB/c mice[37]. As the immunogen for those mice, calf thymus DNA was irradiated with UV-C to exclusively induce CPDs and 6-4PPs. UV-irradiated double-stranded DNA was heat-denatured, electrostatically conjugated with methylated bovine serum albumin, and then emulsified in complete Freund's adjuvant. After immunization, mouse spleen cells were fused with myeloma cells. Culture supernatants from those hybridoma cells were tested for binding specificity to UV-irradiated DNA. We then obtained and characterized six monoclonal antibodies, two TDM and four 64M antibodies as highly specific for CPDs and 6-4PPs, respectively (Table 1). Those antibodies bind to respective types of DNA photoproducts that are in all dipyrimidine sequences (TT, TC, CT and TT) and that occur only in single-stranded DNA. An 8-base long sequence is sufficiently long for stable binding of these antibodies to damaged DNA or oligonucleotides. The nucleotide and amino acid sequence of the variable region of the TDM-2 and the 64M-2, 64M-3, 64M-4, and 64M-5 antibodies were determined and the molecular mechanism of the

Table 1. Monoclonal antibodies specific for DNA photoproducts

Antibody	Antigen	Class	Reference
TDM-1	CPDs	IgM	36
TDM-2[a]	CPDs	IgG_{2a}	37
TDM-3[b]	CPDs	IgG_1	37
64M-1	6-4PPs	IgG_{2b}	34, 35
64M-2[b]	6-4PPs	IgG_{2a}	37
64M-3[b]	6-4PPs	IgG_{2a}	37
64M-4[b]	6-4PPs	IgG_3	37
64M-5[a]	6-4PPs	IgG_1	37
DEM-1	Dewar isomers	IgG_1	38

[a]Simultaneously established from the same mouse.
[b]Simultaneously established from the same mouse.

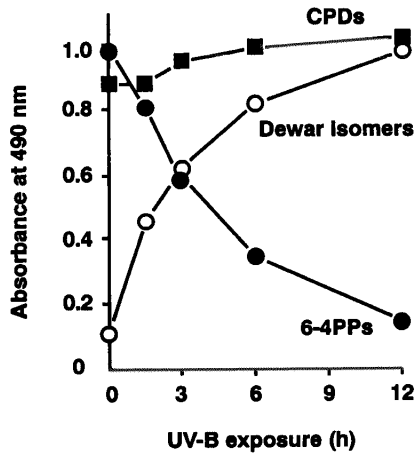

Fig. 2. The kinetics of 6-4PP isomerization to Dewar isomers. Single stranded-DNA was irradiated with 254 nm UV (200 J/m^2) to exclusively induce CPDs and 6-4PPs, and was subsequently irradiated with a Mylar-filtered UV-B light for photoisomerization of 6-4PPs. The modification of antibody binding to 254 nm UV-irradiated DNA by the longer wavelength UV irradiation was determined by an ELISA with TDM-2, 64M-2 and DEM-1 antibodies, which are specific for CPDs, 6-4PPs and Dewar isomers, respectively. The binding of these antibodies was detected with anti-mouse IgG conjugated with peroxidase (for TDM-2 and for 64M-2) or with a biotinylated F(ab')$_2$ fragment of anti-mouse IgG and streptavidin-peroxidase conjugate (for DEM-1) (38).

interaction between DNA photoproducts and these antibodies has been extensively investigated[50]. We prefer to use TDM-2 and 64M-2 antibodies to the others.

We examined the kinetics of 6-4PP photoisomerization to Dewar isomers. The modification of antibody binding to 254-nm UV-irradiated DNA by subsequent exposure to Mylar-filtered UV-B light was determined by ELISA with the TDM-2, 64M-2 and DEM-1 antibodies, which are specific for CPDs, 6-4PPs and Dewar isomers, respectively (Fig. 2)[38]. 254-nm UV exclusively generated CPDs and 6-4PPs, and subsequent Mylar-filtered UV-B irradiation efficiently photoisomerized 6-4PPs and formed Dewar isomers. The ELISA results revealed the specificity of these antibodies to be excellent.

Monoclonal antibodies against thymine dimers have also been generated by Strickland and Boyle[51] and Roza *et al.*[52]. Thymine dimer and 6-4PP monoclonal antibodies are commercially available (Kamiya, WA). Monoclonal antibodies specific for oxidative DNA damage, such as 8-hydroxy-2'-deoxyguanosine and DNA single strand breaks, have also been generated and utilized for studying the photobiology of the skin[2,53]. The 8-hydroxy-2'-deoxyguanosine antibody is commercially available (Genox, MD).

ELISA

We established an ELISA for reliable and sensitive quantitation of CPDs, 6-4PPs and Dewar isomers in DNA[37,38,42,54]. In the case of a DNA repair assay for cultured cells, cellular DNA can be prelabeled with [2-^{14}C]-thymidine in order to avoid the possible influence of DNA replication during the post-UV incubation period (false decrease in the number of

photoproducts per DNA)[42]. Immediately after UV-irradiation or after subsequent DNA repair, genomic DNA is purified from cultured cells or from the skin epidermis and is heat-denatured. 96-well polyvinylchloride flat-bottom microtiter plates, pretreated with 0.003% protamine sulfate, are coated with equal amounts (by weight or radioactivity) of sample DNA. In the case of heavily melanotic cells or epidermis, small amounts of melanin can not be completely excluded from the DNA solutions. In such cases, we prepare DNA solutions that contain the same quantity of melanin (equal to the heaviest melanized sample) by adding a purified melanin solution obtained from a melanosomal fraction to each sample. After blocking, the binding of monoclonal antibodies to photolesions in DNA immobilized in wells is detected with a biotin-streptavidin system. The absorbance of a colored product derived from *o*-phenylene diamine is measured at 490 or 492 nm. For examining repair kinetics, the percentage of the initial number of photolesions (ratio of OD-related UV equivalents) is calculated at various times after UV irradiation using standard damage induction curves, obtained from DNA samples purified immediately after UV irradiation. The formation and repair of CPDs and 6-4PPs can be measured in cultured cells exposed to biologically relevant, sublethal doses of UV, for example 0.5-2 J/m^2 UV-C for CPDs and 5-20 J/m^2 UV-C for 6-4PPs[37]. The difference in UV doses required for CPD and 6-4PP quantitation may be due to the minor yield of 6-4PPs compared with the higher yield of CPDs. We can also measure the formation of CPDs and 6-4PPs in cultured cells exposed to 100-300 J/m^2 of UV-B. The sensitivity of the ELISA assay depends on the dilution of the specific antibodies and the amount of immobilized DNA per well of the microtiter plate, for example, TDM-2 (1/3,333 and 50 ng for 0.5-2 J/m^2 UV-C; 1/1,000 and 15 ng for 5-20 J/m^2 UV-C and 100-300 J/m^2 UV-B) and 64M-2 (1/1,000 and 150 ng for 5-20 J/m^2 UV-C and 100-300 J/m^2 UV-B). Ford and Hanawalt[39] adapted TDM-2 and 64M-2 antibodies to an immunoblot assay and obtained a similar sensitivity. The sensitivity of this ELISA technique is comparable to that of radioimmunoassay[21,26], autoradiography[22], another ELISA[24], high-performance liquid chromatography[25] and immunoslot blot[27] assays using antisera. The sensitivity is also similar to other methods that employ two-dimensional paper chromatography[9], high-performance liquid chromatography[12], comet assay[3], agarose gel electrophoresis[15] or a sucrose gradient[13,14] using a UV endonuclease. There are two sub-pathways of NER, the transcription-coupled repair pathway and the global genome repair pathway. At present, our ELISA technique can determine only global genome repair capacity, while Ford and Hanawalt succeeded in measuring transcription-coupled repair capacity using an endonuclease sensitive site assay[39].

Using this ELISA technique, we compared the induction of CPDs and 6-4PPs in two melanoma cell lines with different levels of pigmentation[48]. Those melanoma cells had sufficient melanin that some was located over nuclei in the path of the incoming UV light, and thus a photoprotective effect of the overlying melanin against DNA damage was expected. The more highly melanotic cell line (HM3KO) formed less CPDs and 6-4PPs than did the less melanotic cell line (Mewo), indicating that melanin reduces DNA photoproduct formation in cultured pigmented cells (Fig. 3). The protection factor achieved by melanin in HM3KO cells was 2.3-2.4 against CPD and 6-4PP formation.

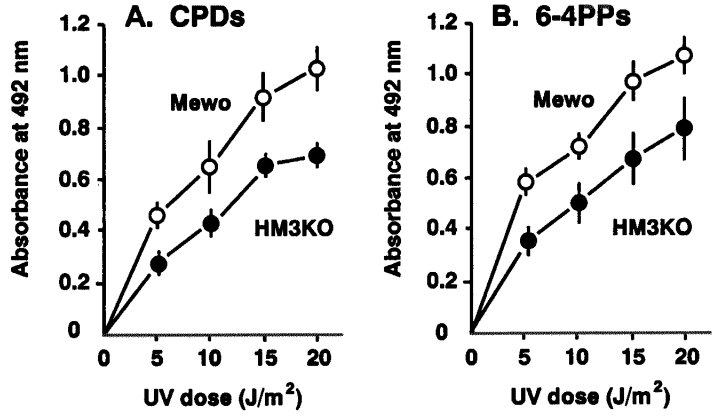

Fig. 3. Melanin reduces DNA photoproduct formation in cultured human melanoma cells. The induction of CPDs (A) and 6-4PPs (B) in DNA from 254 nm UV-irradiated melanoma cells containing different concentrations of melanin (HM3KO 5.2 μg/mm³; Mewo 0.6 μg/mm³) was measured by ELISA with the TDM-2 and 64M-2 antibodies. Each point shows the mean ± standard deviation (SD) of six determinations (48).

INDIRECT IMMUNOFLUORESCENCE IN CULTURED CELLS

We also used these DNA damage-specific antibodies with indirect immunofluorescence to visualize the induction and repair kinetics of DNA photoproducts. The specific fluorescent intensity shows the amount of photolesions in each nucleus, which can be measured by laser-scanning confocal microscopy (laser cytometry)[40-42]. Immediately after UV irradiation or after post UV-incubation to allow DNA repair, cells were treated with ice cold buffer (containing 0.5% Triton X-100, 0.2 mg/ml EDTA and 1% bovine serum albumin in PBS) for 15 min and were then fixed with methanol:acetone (1:1) for 10 min at -20°C. Cells are then treated with 0.07 M NaOH in 70% ethanol for 3 min to denature the DNA. After blocking, the binding of monoclonal antibodies to respective photolesions in DNA within each nucleus can be detected with a biotin-streptavidin system and fluorescein isothiocyanate (FITC). Nuclei are counterstained with propidium iodide (PI). Cells are mounted in drops of an antifade solution and coverslipped. FITC (DNA photoproducts) and PI (DNA) fluorescence is observed and analyzed using a confocal microscope and a computer. The formation of CPDs or 6-4PPs in each nucleus is calculated by dividing the intensity of FITC immunofluorescence by the intensity of PI fluorescence (FITC/PI), because nuclei contain different amounts of DNA. Three-dimensional reconstruction can also be generated from original images with a computer system[42]. The experimental variation in the immunofluorescent laser cytometry is a little larger than that in the ELISA, but it is sensitive enough to determine the induction and repair of CPDs and 6-4PPs in cultured cells after 2-20 J/m² of UV-C irradiation[40,42]. The sensitivity of the quantitative laser cytometric method is similar to that of a qualitative method using indirect immunofluorescence and an antiserum to thymine dimers[21].

We visualized the repair kinetics of CPDs and 6-4PPs in normal human skin fibroblasts

exposed to 30 J/m^2 UV-C in 3-dimensions[42]. Fig. 4 shows reconstructed three-dimensional images of the lower half of nuclei to observe their interiors. These fibroblasts repaired CPDs slowly (Fig. 4A) and 6-4PPs very quickly (Fig. 4B). Most of the 6-4PPs were removed within 3 h after UV irradiation. This result is consistent with the repair kinetics determined by ELISA, in which only ~25% of CPDs were repaired within 6 h but more than 90% of 6-4PPs were removed within 3 h after UV irradiation (Fig. 4C). Interestingly, the punctate, not diffusely spread, nuclear localization of unrepaired 6-4PPs was observed at 2 h after UV irradiation, suggesting that DNA damage may be excised non-randomly from a nucleus during the NER process (Fig. 4B).

We have recently developed a novel technique that uses a micropore filter mask to produce UV-induced DNA damage in localized areas of the cell nucleus[55]. This micropore UV irradiation technique, combined with fluorescent antibody labeling, has enabled us to visualize not only the localized repair of DNA damage but also the recruitment of NER proteins at damage sites. Indeed, we have found that two types of NER proteins, PCNA and

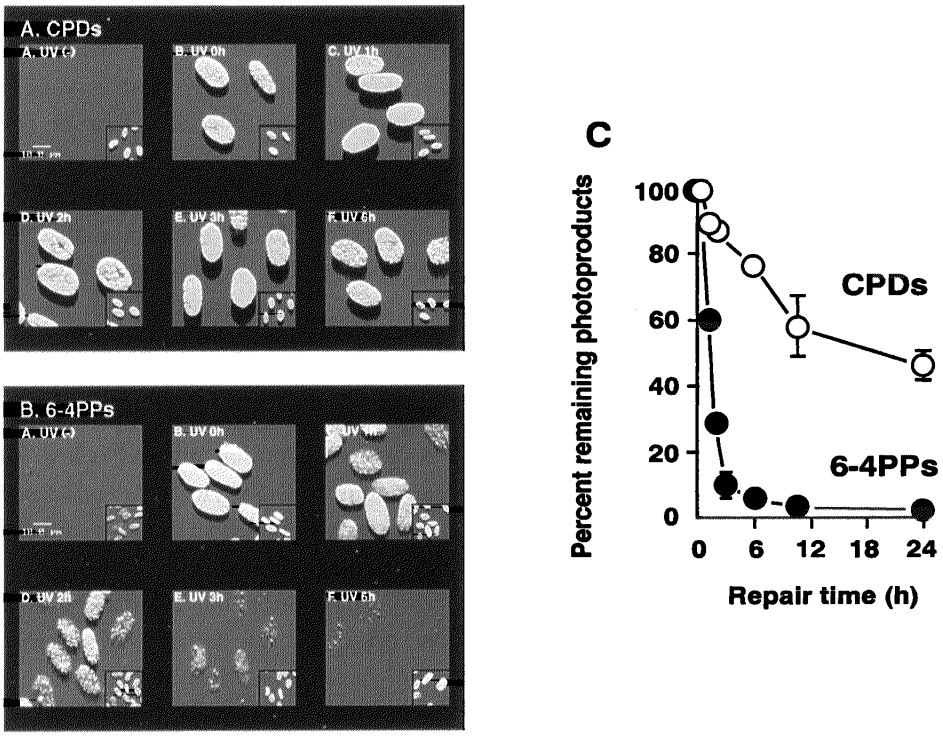

Fig. 4. The repair kinetics of DNA photoproducts in normal human skin fibroblasts. Cells were 254 nm UV-irradiated with 30 J/m^2 and incubated for various times to allow DNA repair. CPDs (A) and 6-4PPs (B) in nuclei were detected by indirect immunofluorescence with TDM-2 and 64M-2 antibodies, respectively, and FITC. The specific fluorescence of CPDs, 6-4PPs and DNA (PI) was obtained and three-dimensional images of the lower half of nuclei were reconstructed based on ~25 consecutive images taken at 0.2 μm intervals using a Meridian InSIGHTplus-IQ laser-scanning confocal microscope and computer system (higher magnification shows CPDs or 6-4PPs, while the lower magnification shows DNA). (C) The repair kinetics were also determined by ELISA with the TDM-2 and 64M-2 antibodies. Each point (±SD) shows the mean of three to four determinations. Scale bar = 10 μm.

DDB2, translocate to the damaged DNA sites[55,56]. Thus, the technique provides a powerful approach to understanding the temporal and spatial interactions between DNA damage and damage-binding proteins in vivo.

INDIRECT IMMUNOFLUORESCENCE IN THE SKIN

In situ immunofluorescent laser cytometry allows one to simultaneously examine DNA photoproducts and histology in multilayered epidermis[43]. It is important to strike a proper balance between fixation and permeabilization of the skin tissue for immunofluorescent staining of DNA photoproducts especially in the skin, because DNA is packed with chromosomal proteins in the nucleus and because TDM-2 and 64M-2 antibodies can bind to CPDs and 6-4PPs only in single-stranded DNA.

Immediately after exposure to UV-B or to solar-simulated radiation, or after DNA repair, skin biopsies are taken, and 3-μm thick cryosections are fixed with 0.001% paraformaldehyde for 3 min at 4℃ and dehydrated through a graded methanol series. Sections are microwaved (500 W) for a total of 5 min in 10 mM citrate buffer, pH 6.0, and then are treated with 0.1% trypsin for 30 min at room temperature. Slides are subsequently treated with 0.07 M NaOH in 70% ethanol for 15 min at room temperature to denature DNA. In the case of organ-cultured skin, or skin explants whose structures are not very firm and are insensitive to trypsinization compared to freshly biopsied skin, stronger fixation may be necessary. Cryosections can be fixed with 4% paraformaldehyde for 30 min, microwaved and treated with 0.1% trypsin for 60 min. After blocking and RNase A treatment, slides are incubated with TDM-2 at 1:10,000 dilution or with 64M-2 at 1:1,500 dilution with 5% fetal bovine serum at 4℃ overnight. The binding of monoclonal antibodies to respective photolesions in each nucleus can be detected with a biotin-streptavidin system and FITC. The nuclei are then counterstained with PI. We can visualize and measure CPDs and 6-4PPs in human or murine skin after irradiation with biologically relevant, suberythematous doses, for example 125-250 J/m^2 UV-B or 0.5-1 MED solar-simulated radiation using this laser cytometric method. Potten *et al.* and Young *et al.* developed a similar method using TDM-1 and 64M-2 antibodies and immunohistochemistry with similar sensitivity[44,57]. The sensitivity of this quantitative laser cytometric method is comparable to that of qualitative immunofluorescence with a polyclonal antibody to pyrimidine dimers[31].

Fig. 5 shows CPD formation in human skin exposed to 1 MED solar-simulated radiation. CPDs were evident in the epidermis and to a lesser extent, in the dermis. UV energy that penetrates to a particular depth in the skin can be estimated by measuring the formation of DNA photoproducts in nuclei at that depth.

Using that approach, we examined the role of supranuclear melanin caps in photoprotection against DNA photoproduct formation in human epidermis[43]. The formation of CPDs was compared in epidermal cells with or without supranuclear melanin caps after UV-B irradiation (Figs. 6A, B). The intensities of CPD immunofluorescence in cells with supranuclear caps were weaker than intensities in cells without supranuclear caps, suggesting that cells with melanin had less DNA damage (detected as CPDs) than did cells without melanin. We measured the formation of CPDs as UV dose equivalents in epidermal cells with or without supranuclear caps located in the same layer of the epidermis in 20

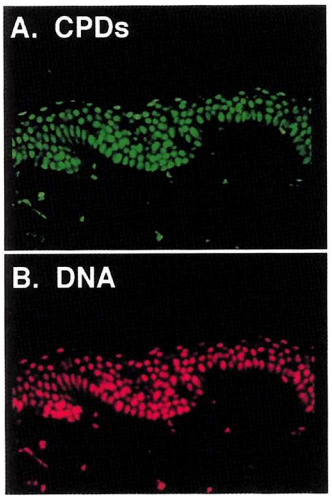

Fig. 5 CPD formation in human skin exposed to 1 MED solar-simulated radiation. A normal human skin sample was biopsied from the lower back of a fair-skinned individual exposed to 1 MED solar-simulated radiation under informed consent. The formation of CPDs was detected by the indirect immunofluorescence using the TDM-2 monoclonal antibody. The FITC immunofluorescence of CPDs (A) and the PI fluorescence of DNA (B) was photographed under a fluorescence microscope. (x 60)

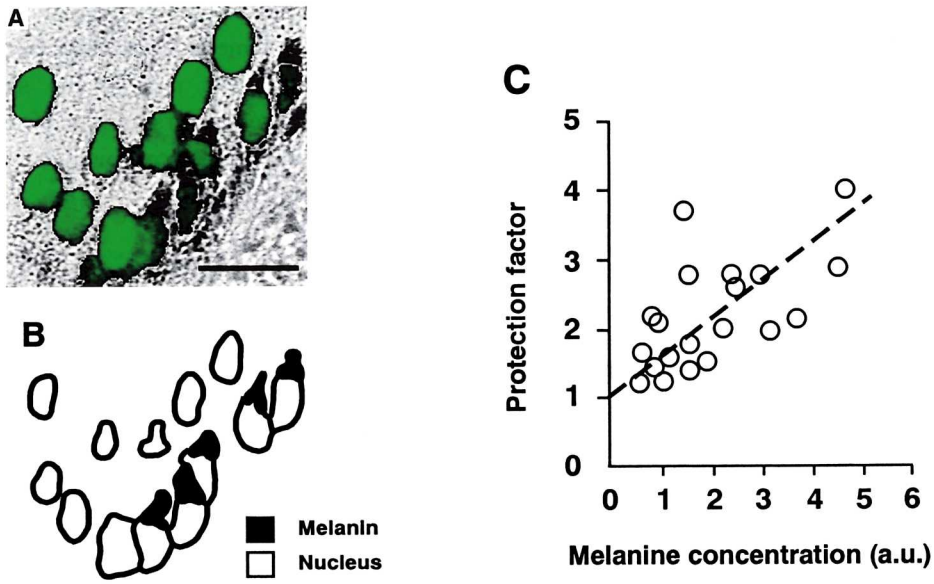

Fig. 6 Supranuclear melanin caps reduce UV-induced DNA photoproducts in a melanin concentration-dependent manner in human epidermis. Normal human skin was biopsied from the lateral side of the left upper arm of a 69-year-old man (skin phototype III, slightly pigmented) under informed consent. The skin explant was exposed to 8000 J/m^2 UV-B to measure very low numbers of CPDs in basal and suprabasal cells with supranuclear caps. The formation of CPDs was compared in epidermal cells with or without supranuclear melanin caps using indirect immunofluorescence with the TDM-2 antibody. (A) The specific fluorescent image is superimposed on the corresponding histological image obtained with transmitted light. (B) Schematic of the image in panel A. (C) Protection factor against CPD formation by supranuclear melanin caps was calculated by dividing the CPD detected in cells without supranuclear caps by that detected in cells with supranuclear caps (ratio of OD-related UV equivalents) in the same layer of the epidermis, in 20 different areas. Melanin concentrations in the supranuclear caps were determined as arbitrary units (a.u.) by measuring their absorbance using the confocal microscope and computer system utilizing an optical microscopic light source (Fig. 6C). Scale bar = 20 μm.

areas. We then calculated the protection factor against CPD formation by epidermal melanin. The protection factor values calculated ranged from 1.15-3.92, which correlated well with melanin concentration (Fig. 6C). We obtained comparable results for 6-4PPs (data not shown). These results indicate that supranuclear melanin caps reduce UV-induced DNA photoproducts in a melanin concentration-dependent manner.

CONCLUSION

A sensitive method for quantitating and visualizing specific types of DNA damage induced by biologically relevant doses of UV or sunlight is essential to understanding UV-induced DNA damage and its biological consequences. An immunological method is the best possible approach for this purpose and monoclonal antibodies are the best probes. We have established monoclonal antibodies that are specific for CPDs, 6-4PPs or Dewar isomers. Those antibodies allow us to quantitate these different types of photoproducts that are generated in DNA by sublethal or suberythematous doses of UV or solar simulated radiation. Various methods, such as an ELISA or laser cytometry, can then be used to visualize such damage by immunofluorescence in cultured cells and in the skin. The techniques described in this review should contribute to a better understanding of molecular mechanisms of cellular responses to UV and DNA damage in pigment cell biology.

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