Melanocytes are deficient in repair of oxidative DNA damage and UV-induced photoproducts

Hsiang-Tsui Wang¹, Bongkun Choi¹, and Moon-shong Tang²

Department of Environmental Medicine, Pathology and Medicine, New York University School of Medicine, Tuxedo, NY 10987

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Melanomas occur mainly in sunlight-exposed skin. Xeroderma pigmentosum (XP) patients have 1,000-fold higher incidence of melanoma, suggesting that sunlight-induced “bulky” photoproducts are responsible for melanomagenesis. Sunlight induces a high level of reactive oxygen species in melanocytes (MCs); oxidative DNA damage (ODD) may thus also contribute to melanomagenesis, and XP gene products may participate in the repair of ODD. We examined the effects of melanin on UVA (320–400 nm) irradiation-induced ODD and UV photoproducts and the repair capacity in MC and XP cells for ODD and UV-induced photoproducts. Our findings indicate that UVA irradiation induces a significantly higher amount of formamidopyrimidine glycosylase-sensitive ODD in MCs than in normal human skin fibroblasts (NHSFs). In contrast, UVA irradiation induces an insignificant amount of UvrABC-sensitive sites in either of these two types of cells. We also found that, compared to NHSFs, MCs have a reduced repair capacity for ODD and photoproducts; H₂O₂ modified- and UVC-irradiated DNAs induce a higher mutation frequency in MCs than in NHSFs; and, XP complementation group A (XPA), XP complementation group C, and XP complementation group G cells are deficient in ODD repair and ODD induces a higher mutation frequency in XPA cells than in NHSFs. These results suggest that: (i) melanin sensitizes UVA in the induction of ODD but not bulky UV photoproducts; (ii) the high susceptibility to UVA-induced ODD and the reduced DNA repair capacity in MCs contribute to carcinogenesis; and (iii) the reduced repair capacity for ODD contributes to the high melanoma incidence in XP patients.

DNA repair and mutagenesis | UV photoproducts | ultraviolet light | melanoma | xeroderma pigmentosum

It has long been recognized that sunlight exposure is the major cause of skin cancers, including melanoma (1–3). Although evidence from both epidemiological and molecular studies show that photoproducts induced by UVB (290–320 nm) irradiation are the major cause for nonmelanoma skin cancer (4), epidemiological studies strongly implicate UVA (320–400 nm) irradiation in sunlight exposure-related cutaneous melanomagenesis (5, 6). In animal models, it has been found that both UVA and UVB irradiation can trigger melanocytic hyperplasia and melanomagenesis (7–9). Although UVB radiation can induce both cyclobutane pyrimidine dimers (CPDs) and pyrimidine <6–4> pyrimidone photoproducts (<6–4> PPs), which can trigger mutagenesis and carcinogenesis (10, 11), the photoproduct yield of these types of DNA damage by UVA irradiation is two to three orders of magnitude lower than by UVB radiation (12). Thus, exactly how UVA acts to promote melanomagenesis remains controversial. It has been found that XP patients have 1,000-fold higher incidence of melanoma than normal individuals (13). Xeroderma pigmentosum cells are sensitive to UV radiation but resistant to ionizing radiation, and XP gene products are also crucial for CPD and <6–4> PP repair (14). Therefore, it is possible that UVA irradiation of melanocytes (MCs) may induce “bulky” photoproducts that are repairable by a nucleotide excision repair (NER) mechanism and these photoproducts, if not repaired, trigger mutagenesis and, consequently, melanomagenesis.

It is well established that the major effect of UVA irradiation is the induction of reactive oxygen species (ROS) and that melanin can greatly enhance this process (15–17). It has been proposed

that UVA-induced oxidative stress (OS) and oxidative DNA damage (ODD) are the two major contributors to UVA-induced melanomagenesis (18). The occurrence of mucosal melanoma in regions that are rarely exposed to sunlight—such as genitalia, the colon, and the nasal septum (19–21)—suggests that DNA damage other than photoproducts is also involved in melanomagenesis. If we hypothesize that ODD is the culprit responsible for triggering mucosal melanomagenesis, then one has to assume MCs in these tissues are undergoing endogenous OS and generating ROS, even without sunlight irradiation. Because melanin is an effective free radical scavenger and would presumably mitigate OS, the role of melanin in melanomagenesis remains unclear (22).

Previously, using host cell reactivation (HCR) and in vitro repair synthesis assays, we have found that OS and lipid peroxidation (LPO) byproducts—such as 4-hydroxy-2-nonenal (4-HNE), malondialdehyde (MDA), and acrolein (Acr)—inhibit NER and enhance mutagenesis through direct modification of repair proteins by the carbonyl group in the aldehydes (23–25). These findings raise the possibility that the intrinsically high OS in melanocytes and OS induced by UVA irradiation may induce LPO; LPO byproducts could then reduce the DNA repair capacity, which in turn could contribute to melanomagenesis.

To understand the role of melanin in UVA radiation-induced DNA damage formation and the mechanism that leads to high melanoma incidence in XP individuals, we determined the effects of melanin on UVA-induced ODD and UV photoproduct formation; the repair capacity for ODD and UV photoproducts in MC, XPA, XPC, and XPG cells; and the mutagenicity of ODD in these cells. Our results show that melanin plays a crucial role in UVA-induced mutagenesis and also, most likely, in melanomagenesis.

Results

Oxidative DNA Damage and Bulky Photoproducts Induced by UVA Irradiation in MCs. It has been shown that melanin enhances free radical generation, including reactive ROS resulting from UVA irradiation (15–17). Hence, it is possible that melanin enhances ODD. To test this possibility, lightly and darkly pigmented MCs and normal human skin fibroblasts (NHSFs, CRL2097) were irradiated with UVA light and the formation of ODD was detected by the formamidopyrimidine glycosylase (Fpg) incision assay. Formamidopyrimidine glycosylase incises 8-oxo-deoxyguanosine (8-oxo-dG) and the imidazole ring-opened adducts of purines; these adducts are generated in cells under OS and also in H₂O₂-treated DNA (26). The results in Fig. 1A show that UVA irradiation does indeed enhance Fpg-sensitive site formation in both

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¹H.-T.W. and B.C. contributed equally to this work.

²To whom correspondence should be addressed. E-mail: moon-shong.tang@nyumc.org.

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induced DNA damage between these two types of MCs, raise the possibility that TCR for UV damage is defective in lightly pigmented, but not darkly pigmented, MCs.

The results in Fig. 2*B* show that the capacity of MCs to reactivate a H_2O_2 -modified luciferase gene is significantly lower than the capacity of NHSFs. Darkly pigmented MCs have a lower repair capacity than lightly pigmented MCs. It appears that the reduction of the expression of the H_2O_2 -modified luciferase gene is biphasic in NHSFs, indicating that there are two pathways for repair of H_2O_2 -induced DNA damage. The differences between NHSFs and MCs are present only at the initial phase for low levels of H_2O_2 -induced DNA damage. The sharp slope at the initial phase indicates that melanin affects the major repair pathway for ODD. The results in Fig. 2*B* and *C* show that XPA, XPC, and XPG cells have a repair capacity comparable to that of NHSFs for low levels of DNA damage induced by H_2O_2 modifications but are deficient in repair of higher levels of H_2O_2 modification. The results suggest that XPA, XPC, and XPG cells are deficient in a repair pathway operating on DNA damage induced by high levels of H_2O_2 modification.

Cell Lysates of MCs Have a Lower Capacity to Mediate DNA Damage-Induced Repair Synthesis. The HCR assay of the damaged luciferase gene measures the repair capacity for the transcriptionally active luciferase gene. This may or may not represent the host cell repair capacity for the overall genome, which would also include the nontranscribed strand and noncoding genomic regions. To resolve this issue, we measured the capacity of cell lysates to carry out DNA damage-induced repair synthesis. We found that the level of in vitro DNA repair synthesis is proportional to the amount of UV photoproducts or ODD in the DNA substrates, which validates the use of this system for measuring the DNA repair capacity (Fig. S5). The results in Fig. 3 show that MC cell lysates have a lower capacity to carry out both photoproduct- and ODD-induced repair synthesis than NHSF cell lysates. Darkly pigmented MC lysates have a lower capability for repair synthesis induced by both UV photoproducts and ODD than lightly pigmented MC cell lysates. The results in Fig. 3 also show that cell lysates from

XPA cells have the lowest capacity for repair synthesis induced by either type of DNA damage. These results suggest that the XPA protein is involved in repair of ODD as well UV photoproducts.

Melanin Interferes with in Vitro DNA Damage Specific-Repair Synthesis.

There are two possibilities to account for the reduced HCR capacity in MCs and the reduction in DNA damage-induced repair synthesis in MC cell lysates: (i) melanin directly interferes with excision repair, and/or (ii) melanin suppresses the expression of repair genes. To test the first possibility, the effect of melanin on in vitro DNA damage-induced repair synthesis was determined. Different amounts of melanin were added directly to cell lysates isolated from NHSFs and the capacity for DNA repair synthesis in these cell lysates was then determined. Results in Fig. 4 show that the addition of melanin reduced repair synthesis induced by both photoproducts and ODD; moreover, the extent of repair reduction was proportional to the amount of melanin added to the cell lysates.

To determine how melanin interferes with DNA repair, different amounts of melanin were added to UVC-irradiated or H_2O_2 -modified NHSF genomic DNA and these DNA-melanin mixtures were then tested for their sensitivity toward the NER enzyme UvrABC and the base excision repair (BER) enzyme Fpg. The results shown in Fig. 5 reveal that the addition of melanin to the UVC-irradiated or H_2O_2 -modified DNA indeed reduced its sensitivity toward UvrABC or Fpg incision, respectively. During genomic DNA isolation from MCs, it was found that melanins copurified with genomic DNAs and the color of the genomic DNAs was dark. These darkly colored genomic DNAs were resistant to UvrABC and Fpg. However, when genomic DNAs were separated from melanin-DNA complexes by electrophoresis, the genomic DNAs became sensitive to UvrABC and Fpg incision (Fig. S6). These results strongly suggest that melanin binding renders the DNA refractory to recognition and incision by NER and BER enzymes.

UVC-Irradiated or H_2O_2 -Modified *supF* Gene Has a Higher Mutation Rate in MCs Than in NHSFs. Results from both HCR and in vitro repair synthesis assays indicate that MCs have a lower repair ca-

Fig. 3. Determination of the capacity of cell lysates isolated from NHSF, MC, and XPA cells in mediating DNA damage-induced repair synthesis. Cell lysates were isolated from exponentially growing cells and used to mediate repair synthesis using (A) UV-damaged or (B) H_2O_2 -modified pUC18 plasmid and undamaged control pBR322 plasmid as substrates in the presence of $\alpha\text{-}^{32}\text{P}$ dATP. The resultant plasmid DNAs were purified, digested with HindIII, and separated in a 1% agarose gel by electrophoresis. In the *Top* panel are typical photographs of ethidium bromide-stained gels, and in the *Middle* panel are the autoradiographs of the same gels. The relative repair capacity was calculated based on the ratio of the amount of repair synthesis over the amount of substrate DNA (*Bottom*). Symbols are the same as in Figs. 1 and 2. UVC dose: 1,500 J/m², H_2O_2 modification: 100 mM for 30 min at 37 °C. The data are from three independent experiments. *P* values for HEMn-DP vs. HEMn-LP are 0.03 for UVC-induced DNA damage and 0.09 for H_2O_2 -induced DNA damage.

Fig. 4. Effect of melanin on DNA damage-induced repair synthesis mediated by NHSF cell lysates. DNA damage-induced repair synthesis was carried out by NHSF cell lysates (six lanes from left in A and B), the same as described in Fig. 3, except different amounts of melanin were added to the NHSF cell lysates. In A, UVC (1,500 J/m²)-irradiated DNA was used as the substrate, and in B, H₂O₂- (100 mM, 30 min at 37°C) modified DNA was used as the substrate. In HEMn-LP lane, the repair DNA synthesis was carried out by cell lysates of lightly pigmented melanocytes. The symbols are the same as in Fig. 3. The quantitative data in C are from three independent experiments.

capacity for UV photoproducts and ODD. These results raise the possibility that MCs are more susceptible to DNA damage-induced mutagenesis. To date no mutation assay has been established to measure DNA damaging agent-induced mutagenesis in normal human melanocytes. Therefore, a human-*Escherichia coli* shuttle vector system was used to determine the effects of reduced repair capacity on DNA damaging agent-induced mutagenesis. Plasmid DNA containing *supF* was modified with H₂O₂ (100 mM, 30 min at 37 °C) or irradiated with UVC (1,500 J/m²) and then transfected into MCs. After 72 h of incubation, the replicated plasmid DNAs were isolated and the mutations in the *supF* gene determined using indicator *E. coli* cells. The results in Table 1 show that the H₂O₂-modified plasmid DNA induces a 7- to 19-fold higher mutation frequency in MCs than in NHSFs, and UVC-irradiated plasmid DNA induces a 7- to 14-fold higher mutation frequency in MCs than in NHSFs. Both H₂O₂- and UVC-induced

DNA damage induce a higher mutation frequency in darkly pigmented MCs than in lightly pigmented MCs. The results also show that H₂O₂-modified and UVC-irradiated plasmid DNAs induce a 5-fold and a 52-fold higher mutation frequency, respectively, in XPA cells than in NHSFs. These results are consistent with the findings that demonstrated that MC and XPA cells are defective in ODD and UV photoproduct repair and that darkly pigmented MCs are more defective in these repair pathways than lightly pigmented MCs.

Discussion

Skin cancers, including cutaneous melanoma, occur mainly in body regions exposed to sunlight, clearly indicating that sunlight-induced DNA damage plays a major role in skin carcinogenesis (1–6). It is generally accepted that sunlight in the UV range of 290–400 nm is the primary cause of the DNA damage that consequently triggers mutagenesis and carcinogenesis (10, 11, 29). Although shorter wavelength (290–320 nm, UVB) irradiation can induce the formation of CPDs and <6–4> PPs in DNA, the yield of these photoproducts in the UVA region (320–400 nm) is likely very low, if not zero (12). Therefore, the role of UVA in skin carcinogenesis, particularly in melanomagenesis, remains controversial. Because skin cells absorb far more energy from UVA than UVB and UVA can penetrate deeper than UVB (35), it is imperative to understand the biochemical effects of UVA irradiation to better assess risk and to design effective measures for skin cancer prevention.

UVA irradiation has been shown to induce free radicals, including ROS, and melanin enhances this effect (15–17). Moreover, it is well established that OS induces LPO and that the byproducts of LPO—such as HNE, MDA, and Acr— inhibit DNA repair (23–25). Consistent with these results, we found that UVA irradiation induces OS and ODD in MCs (Fig. 1), MCs have a lower repair capacity for UV photoproducts and ODD (Fig. 2), and ODD and UV photoproducts induce more mutations in MCs than in NHSFs (Table 1). Together, these results lead us to propose that UVA-induced, melanin-augmented ODD in MCs and the inherently reduced repair capacity in these cells are the two

Fig. 5. Effect of melanin on Fpg and UvrABC incision activity. Genomic DNA (1 μg) isolated from NHSF cells was modified with H₂O₂ (10 mM, 2 h at room temperature) or irradiated with UVC (20 J/m²), incubated with different amounts of melanin (0, 5, 20, 100, 200, and 400 ng) in TE buffer (final volume 100 μL) for 2 min and then reacted with (A) Fpg for H₂O₂-modified DNA or (B) UvrABC for UVC-irradiated DNA. The Fpg and UvrABC reaction conditions and the separation of the resultant DNAs were the same as described in Fig. 1.

Table 1. Mutagenicity of H₂O₂- and UVC-induced damage in the *supF* gene in NHSF (CRL2097), MC (HEMn-LP and HEMn-DP) and XPA cells

Cell type	Treatment*	Exp.	No. of mutants/ no. of total colonies	Mutation frequency ($\times 10^4$)	Fold change	P value
CRL2097	Control	1	9/48,570	1.85	1.0	
	UV	1	8/16,698	4.79	2.6	
	H ₂ O ₂	1	13/59,682	2.18	1.2	
HEMn-LP	Control	1	2/17,626	1.13	1.0	
		2	2/13,004	1.54	1.0	
	UV	1	9/8,542	10.54	9.3	0.00050
		2	6/5,649	10.62	6.9	
	H ₂ O ₂	1	12/11,002	10.91	9.6	0.00320
		2	10/10,042	9.96	6.5	
HEMn-DP	Control	1	3/12,858	2.33	1.0	
		2	3/13,428	2.23	1.0	
	UV	1	33/9,864	33.45	14.3	0.00271
		2	21/6,916	30.36	13.6	
	H ₂ O ₂	1	50/11,546	43.31	18.6	0.00020
		2	36/8,538	42.16	18.9	
XPA	Control	1	3/19,344	1.55	1.0	
	UV	1	56/6,900	81.16	52.3	
	H ₂ O ₂	1	18/22,800	7.89	5.1	

Exp., experiment.

*UV: UVC, 1.500 J/m²; H₂O₂: 100 mM, 30 min at 37 °C.

key factors that contribute to cutaneous melanomagenesis. It is worth noting that mucosal melanoma occurs in regions rarely exposed to sunlight; i.e., the colon, genital organs, and the nasal septum (19–21); these particular melanomas most likely are not induced by UV photoproducts. Our results suggest that the inherently low repair capacity in MCs may contribute to melanomagenesis in these tissues.

Melanoma incidence in XP patients is 1,000-fold higher than in normal individuals (13). It is well established that XP cells are sensitive to UVC irradiation-induced cell killing and that XP gene products recognize and repair UV photoproducts and bulky chemical DNA damage. In contrast, compared to normal human fibroblasts, XP cells are not sensitive to ionizing radiation-induced cell killing (14). Therefore, it is believed that XP proteins are involved in NER, but not BER (14). Because ample epidemiological studies have indicated that UVA irradiation is related to melanoma incidence, to account for the high incidence of melanoma in XP patients it has been proposed that melanin sensitizes cells to UVA irradiation, resulting in the generation of "bulky" photoproducts, which are repaired by the NER mechanism. Because XP patients are deficient in the repair of these bulky photoproducts, XP individuals are more prone to develop melanoma. Our results show that XPA, XPC, and XPG cells are deficient in the repair of both bulky photoproducts and ODD and that both types of DNA damage induce more mutations in XPA cells than in NBSF cells (Fig. 2 and Table 1). These results indicate that the XPA gene product is involved in both NER and BER mechanisms. Furthermore, our results show that UVA irradiation does not induce bulky photoproducts. Our findings provide a plausible explanation for why XP patients are more prone to develop melanoma: UVA irradiation induces more ODD and OS in MCs than in fibroblasts and XP individuals are deficient in ODD repair.

It is intriguing that, compared to NHSFs, MCs have lower BER and NER capacity; the underlying mechanisms for this phenomenon are unclear. Previously, we have found that OS and LPO byproducts, such as 4-HNE, MDA, and Acr, can cause an inhibitory effect on NER (23–25). Because melanin is a known ROS scavenger, it is unlikely that without UVA irradiation, MCs would have a higher level of ROS than NHSFs. In fact, we found

that the ROS level in MCs is similar to that in NHSFs. We also found that the addition of melanin to NHSF lysates directly causes an inhibitory effect on repair synthesis and that melanin-associated DNA is refractory to repair enzymes. Hence, it is possible that the inhibitory effect of melanin on DNA repair arises through the interaction of melanin with repair proteins directly and/or with DNA. Because both scenarios could occur in vivo, we propose that reduced DNA repair capacity contributes to melanomagenesis, particularly in mucosal melanomas.

Materials and Methods

Culture of Primary Human Melanocytes and Skin Fibroblasts. Human epidermal melanocytes lightly pigmented (HEMn-LP) and human epidermal melanocytes darkly pigmented (HEMn-DP) were maintained in Medium 254 supplemented with human melanocyte growth supplement (Cascade Biologics, Invitrogen). The amount of melanin in each type of cell was checked by the melanin assay (Fig. S2) (36). Primary culture of normal human skin fibroblasts (NHSFs, CRL2097, American Type Culture Collection) was grown in minimum essential medium (Invitrogen) supplemented with 10% FBS. Human XPA-deficient XPA (GM05509), XPC (GM01736), and XPG fibroblasts (GM16398) (National Institute of General Medical Sciences, Human Genetic Cell Repository) were grown in minimum essential medium supplemented with 15% FBS.

UVA Irradiation and Genomic DNA Isolation. The monolayer ~70% confluent cells were irradiated with different doses (0, 2, and 10 J/cm²) of UVA light (LT18W/009UV) filtered through Mylar polyester film (Du Pont) in PBS buffer. Immediately after irradiation, cells were harvested and the genomic DNA was isolated as previously described (23).

Fpg and UvrABC Incision Assays. Fpg, UvrA, UvrB, and UvrC were isolated from *E. coli* cells as previously described (37, 38). It is well established that the three UvrABC proteins work in concert to incise the UVC photoproducts CPD and pyrimidine < 6-4 > pyrimidone (27, 28), and Fpg can incise oxidative base damage such as 8-oxo-deoxyguanosine (8-oxo-dG) and its open-ring products (26). The Fpg incision assay was carried out in the same manner as described by Boiteux et al. (38). The UvrABC incision assay and the separation of the resultant DNA were carried out as previously described (23).

Host Cell Reactivation, in Vitro Repair Synthesis, and *supF* Mutation Assays. Methods for isolation of plasmid luciferase plasmid pGL3, pSV- β -galactosidase, pUC18, pBR322, and pSP189, the HCR assay, the in vitro repair synthesis assay, and *supF* mutation detection were the same as previously described (23–25).

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