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UV-Induced DNA Damage and Repair: The Role of Melanin and the MC1R Gene

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Abstract

Malignant melanoma is the most lethal skin cancer. Multiple factors influence melanoma susceptibility, such as the melanocortin 1 receptor (MC1R) gene, cutaneous melanin content, and ultraviolet radiation (UVR) exposure. After irradiating cultured human melanocytes with increasing UVR doses, we examined the influence of melanin content and MC1R function on the induction and repair of DNA photoproducts, particularly cyclobutane pyrimidine dimers (CPD). We analyzed constitutive pigmentation by total melanin content as well as by its components, eumelanin and pheomelanin. In melanocytes expressing functional MC1R, total melanin and eumelanin contents correlated inversely with UVR-induced growth arrest, apoptosis, and CPD induction; however, hydrogen peroxide release was positively correlated. Conversely, melanocytes with loss-of-function MC1R, regardless of total melanin or eumelanin contents, sustained higher levels of UVR-induced apoptosis and CPD induction, with reduced CPD repair capacity. Melanin content, primarily eumelanin content, and MC1R genotype are independent determinants of UVR-induced DNA damage in cultured human melanocytes.

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(B) represent the dose-dependent release of hydrogen peroxide from melanocytes with high vs. low EC, respectively.
Introduction

Malignant melanoma is a neoplasm that originates in melanocytes and is the most lethal of all skin cancers. Worldwide data from 2002 indicated that over 160,000 new cases of melanoma were diagnosed, and almost 41,000 deaths resulted from melanoma (Ferlay J, 2002). In the U.S.A., the predicted incidence of epithelial melanoma for 2006 is over 62,000 cases, accounting for approximately 5% of all new cancer cases in males and females. During 2006 alone, melanoma will be responsible for the deaths of almost 8,000 Americans (American Cancer Society, 2006).

Melanoma is a multifactorial disease, and the mechanism of carcinogenesis is not well understood. The accumulation of successive genetic mutations in normal melanocytes leads to neoplastic transformation of these cells (Czajkowski R; Drewa, 2002). Since familial melanoma only accounts for a small percentage of melanoma cases, defining the etiology of sporadic cutaneous malignant melanoma (CMM) is particularly important (Kamb, 1995; Piepkorn, 2000). Sun exposure, particularly during childhood, is an environmental risk factor for melanoma (Armstrong BK, 2001),(Fears TR, 2002). The major and specific type of UVR-induced DNA damage is in the form of DNA photoproducts, primarily CPD (Mitchell et al., 1991). Inefficient repair of DNA photoproducts is associated with melanoma risk, as exemplified by the disease xeroderma pigmentosum (XP) (Kraemer et al., 1994). The XP paradigm incorporates the elements of UVR exposure, DNA damage, and nucleotide excision repair (NER) into the melanoma model. Patients with XP are deficient in NER due to mutations in specific genes for enzymes involved in the NER pathway, and have 1000-fold higher risk of melanoma compared to the general population (Kraemer, 1994). Additional evidence for a role of repair of UVR-induced DNA damage came from a recent study that found melanoma patients inefficient in
repairing UVR-induced DNA damage (Wei et al., 2003). Another study found that mutations in the melanoma susceptibility genes p16 and ARF reduce the capacity to carry out NER, further emphasizing the significance of repair of DNA photoproducts in melanoma susceptibility (Sarkar-Agrawal et al., 2004).

Melanin in the skin, particularly the brown-black pigment, eumelanin, reduces the penetration of UVR through the epidermal layers and quenches reactive oxygen radicals (Kaidbey et al., 1979), (Kobayashi et al., 1998), (Menon et al., 1983), (Bustamante et al., 1993). Pheomelanin, on the other hand, is thought to have poor photoprotective properties due to its propensity to degradation by UVR and its potential to generate reactive oxygen species. In general, melanin content in the skin correlates inversely with the extent of UVR-induced DNA damage and the incidence of skin cancer, including melanoma (Tadokoro et al., 2003), (Epstein, 1983), (Sober et al., 1991). Thus, it is commonly accepted that individuals with the highest risk of skin cancer, including melanoma, are those with skin phototypes I and II, characterized by red hair, fair skin with freckles, and poor tanning ability. However, there is increasing evidence that the risk of melanoma cannot be accurately ascertained based solely on pigmentary phenotype, since there are other independent melanoma risk factors that determine susceptibility.

An independent risk factor for melanoma is the \textit{MC1R} gene, which has emerged as a potential melanoma susceptibility gene. The human \textit{MC1R} codes for the melanocortin 1 receptor, a G\textsubscript{s}-protein-coupled receptor with seven transmembrane domains that is mainly expressed on melanocytes. The MC1R is activated by its ligands \(\alpha\)-melanocortin (\(\alpha\)-MSH) and ACTH, which are known to regulate the synthesis of eumelanin (Mountjoy et al., 1992), (Suzuki et al., 1996), (Hunt et al., 1995). The \textit{MC1R} is considered a major contributor to the diversity of human pigmentation, due to its extensive polymorphisms. So far, more than 65 allelic variants
of the \textit{MC1R} have been found in different populations (Wong, 2005). Certain \textit{MC1R} alleles, particularly R151C, R160W, and D294H substitutions, are strongly associated with the red hair/fair skin phenotype. However, these variants are necessary but not sufficient for this pigmentation phenotype (Box et al., 1997), (Smith et al., 1998). The above three alleles are loss-of-function mutations in the \textit{MC1R}, and are also associated with poor tanning ability and increased risk for melanoma independent of pigmentation phenotype, suggesting a gene-environment interaction between \textit{MC1R} and solar UVR exposure (Scott et al., 2002b), (Ringholm et al., 2004), (Palmer et al., 2000), (Kennedy et al., 2001), (van der Velden et al., 2001), (Matichard et al., 2004), (Landi, 2005). We have recently discovered that activation of the MC1R by its ligands promotes the survival of human melanocytes, enhances their capacity to repair CPD, and counteracts the release of hydrogen peroxide following UVR irradiation (Kadekaro et al., 2005). These effects were evident in tyrosinase-negative albino melanocytes, and absent in human melanocytes expressing loss-of-function MC1R. These novel findings suggest that the MC1R regulates DNA repair mechanisms in addition to its function in the control of eumelanin synthesis in human melanocytes.

In this study, we utilized a panel of cultured human melanocytes, each derived from a single donor, to evaluate the role of MC and EC, as well as \textit{MC1R} genotype, in UVR-induced growth arrest, apoptosis, generation and repair of CPD. To our knowledge, this is the largest \textit{in vitro} study undertaken to provide experimental evidence for a widely accepted theory, based mainly on epidemiological evidence, that MC correlates inversely with UVR-induced DNA damage, and to investigate the role of \textit{MC1R} genotype in the melanocyte response to UVR. Previously, we reported that human melanocytes respond to UVR with growth arrest, cytotoxicity, and induction of DNA photoproducts, and that these effects are more pronounced in
melanocytes with low MC, compared to melanocytes with high MC (Barker et al., 1995).

Another study using cultured human melanocytes from 3 different individuals demonstrated that eumelanin is more photoprotective than pheomelanin, as the former reduced the induction of DNA photoproducts following UVR exposure (Smit et al., 2001). Our present results are derived from an extensive study of 22 different melanocyte cultures and lend strong evidence for a role of EC and MC in limiting the extent of UVR-induced growth arrest, apoptosis, and CPD formation. However, our data indicate that this role is only true in melanocytes with functional MC1R, and is remarkably reduced in melanocytes that express loss-of-function MC1R alleles in the compound heterozygous or homozygous state. The latter melanocytes encounter more CPD and have reduced efficiency to carry out NER than their former counterparts. These findings suggest reduced NER capacity as a potential mechanism by which loss-of-function MC1R alleles contribute to the risk for melanoma.

**Results**

**Total melanin, eumelanin and pheomelanin contents of melanocyte cultures used in the study**

We measured the induction of CPD by 7, 14, 21, and 35 mJ/cm² UVR in 15 neonatal and 3 adult melanocyte cultures, and correlated that with the respective MC and EC (Table 1, Fig. 1). The MC and EC values of some of these cultures were reported in two previous studies (Scott et al., 2002; Wakamatsu et al., 2005). Six of the neonatal melanocyte cultures classified as (L) were lightly pigmented with EC ranging between 0.66 and 4.13 µg/10⁶ cells and MC between 1.09 and 10.07 µg/10⁶ cells. Seven were intermediately pigmented, designated as (D), with EC ranging between 10.64 and 56 µg/10⁶ cells and MC ranging between 17.25 and 67.42 µg/10⁶ cells. The remaining 2 neonatal cultures were darkly pigmented, designated as (D⁺) with EC
values between 76.16 and 100 µg/10^6 cells and MC values between 82.83 and 141.42 µg/10^6 cells (Table 1). Of the total 19 cultures, one (753L) was homozygous for R160W substitution, two were compound heterozygote for R160W and D294H (830L) and R151C and D294H substitutions (849D), and two (1036L and 1201D) were heterozygous for R160W and D294H substitutions in the *MC1R*, respectively. One culture, 1257L was refractory to α-MSH, but expressed wild type *MC1R*, suggesting a mechanism other than expression of *MC1R* loss-of-function allelic variants in abolishing the response to melanocortins. Interestingly, 849D, with loss-of-function *MC1R*, had MC and EC comparable to those intermediately pigmented melanocytes, confirming that loss-of-function *MC1R* variants are not strictly associated with very light skin color. Among the adult melanocyte cultures, AHML, derived from a female Caucasian donor had the lowest MC and EC, as reported previously (Wakamatsu et al., 2005). The second culture established from a female Indian donor (SB) had higher MC and EC, and the remaining culture from an African American female donor had the highest MC and EC (Table 1). Eumelanin content, but not pheomelanin content, correlated directly and consistently with MC, indicating that EC is the major contributor to the diversity of cutaneous pigmentation.

**UVR-induced growth arrest and apoptosis of human melanocytes**

Irradiation of melanocytes with 7, 14, 21, or 35 mJ/cm² resulted in dose-dependent growth arrest and death (Fig. 1). Melanocytes with high MC and EC did not exhibit any decrease in viability or proliferation rate following exposure to the lowest dose of UVR, and showed a slight increase in cell death (around 10%), accompanied with only an initial reduction in proliferation rate on day 2 after irradiation with 14 mJ/cm² UVR (Fig. 1A and C). Marked reduction in viability and proliferation rate was observed in the group irradiated with 21 mJ/cm², which became more pronounced in the group exposed to the highest dose of 35 mJ/cm² UVR. In
comparison, melanocytes with low MC and EC showed no alteration in viability, but a profound reduction in proliferation rate after exposure to 7 mJ/cm² UVR (Fig. 1B and C). Irradiation of these cultures with 14 mJ/cm² UVR resulted in cell death, equivalent to 20, 40 and 30% of total cell number on days 2, 4, and 6 after irradiation, respectively, accompanied by complete growth arrest (Fig. 1B and C). The higher doses of 21 and 35 mJ/cm² UVR further increased cell death up to 70-80% on day 4, with total cessation of proliferation.

Following irradiation with 7, 14, 21 or 35 mJ/cm² UVR, Annexin V staining of apoptotic melanocytes further confirmed the differences in the UVR-induced cytotoxicity in melanocytes with different MC and EC (Fig. 2). Twenty-four hours post irradiation, no apoptosis was detected in melanocytes irradiated with 7 mJ/cm² UVR, regardless of their MC or EC. Irradiation with the higher doses of UVR resulted in dose-dependent increase in apoptosis. Melanocytes with low MC and EC and functional MC1R (1292L) encountered 15, 32, and 45% in apoptosis above control, as opposed to D⁺ melanocytes, responsive to melanocortins and with high MC and EC that exhibited 0, 10, and 29% increase in apoptosis in response to irradiation with 14, 21, or 35 mJ/cm² UVR, respectively. Melanocytes refractory to melanocortins (753L and 1257L) had the greatest increase in apoptosis in response to UVR, which was most evident at doses equal to, or higher than, 21 mJ/cm² (Fig. 2). Irradiation with the highest dose of 35 mJ/cm² UVR resulted in 65% increase in apoptosis in these two melanocyte cultures, compared to 44% increase in melanocytes with functional MC1R and low MC and EC, and 29% increase in melanocytes with functional MC1R and high MC and EC.

We previously reported that UVR-induced apoptosis is accompanied by reduction in the level of Bcl2, known to be critical for melanocyte survival (Im et al., 1998), (Tada et al., 1998), (Kadekarro et al., 2005), (Veis et al., 1993). Thus, we determined the effects of 7, 14, 21, and 35
mJ/cm² UVR on Bcl2 levels, and correlated these effects with the increase in apoptosis (Fig. 3). A dose-dependent decrease in Bcl2 levels was detectable 24 h after irradiation in the groups exposed to the highest two doses of UVR.

**Effects of MC, EC and MC1R on Induction of CPD**

The levels of CPD were determined by Southwestern blot analysis of DNA isolated from melanocytes immediately after irradiation with 7, 14, 21, or 35 mJ/cm² UVR. A dose-dependent increase in CPD was evident in all the cultures tested, irrespective of their MC or EC (Fig. 4). However, marked differences in CPD levels were observed in melanocyte cultures with varying MC and EC. In the panel of neonatal melanocyte cultures, the highest levels of CPD were induced in melanocytes with the lowest MC and EC, intermediate levels of CPD were induced in melanocytes with intermediate MC and EC, and the least CPD were generated in melanocytes with highest MC and EC (Fig. 4A, B). In response to the lowest dose of 7 mJ/cm² UVR, melanocyte cultures with the least MC and EC sustained 125% increase in CPD, which increased to 190% following irradiation with 35 mJ/cm² UVR. In comparison, the levels of CPD in the intermediately pigmented cultures increased to 50% above control in response to 7 mJ/cm² UVR and reached 125% after irradiation with 35 mJ/cm². In the most pigmented cultures, induction of CPD increased gradually from 10-65% above control in response to increasing doses of UVR (Fig. 4A, B). Linear regression analysis after Box-Cox transformation of the response variable, CPD, for these cultures (excluding 830L and 849D) showed that most regression coefficients were highly statistically significant (p-values for 7, 14, 21, and 35 mJ/cm² UVR were 0.0086, 0.0558, 0.0202, and 0.0102, respectively), indicating that eumelanin affects CPD induction. Adult melanocytes (n=3) showed a similar trend as neonatal melanocytes, with the Caucasian-derived melanocytes (AHML) incurring 120% more damage than controls at UVR doses equal to
or higher than 14 mJ/cm² (Fig. 4C). In comparison, melanocytes derived from an Indian donor (SB) had a maximal level of CPD equivalent to 80%, and African-American-derived melanocytes (AHMD⁺) had a maximum of only 50% increase in CPD levels, above control. These results indicate that MC and EC correlate inversely with the induction of DNA photoproducts.

We also investigated the role of MC1R in the induction of CPD in melanocytes. We observed that among all the melanocyte cultures tested, the highest levels of CPD were induced in melanocytes with loss-of-function MC1R, regardless of their MC or EC. One culture (849D), compound heterozygous for R151C and D294H, and with MC and EC of 16.72 and 14.90 μg/10⁶ cells, respectively, had markedly higher levels of CPD than any of the lightly pigmented cultures with functional MC1R. In this culture, the maximal level of CPD generated in response to 35 mJ/cm² UVR was 250% above control, compared to 150% increase in melanocytes with the lowest melanin content and functional MC1R. Another culture, 830L, compound heterozygous for R160W and D294H and with low MC and EC, incurred the maximal level of CPD of all the cultures tested, reaching 375% above control in response to 35 mJ/cm² UVR. Melanocytes with functional MC1R and similar MC and EC (1292L and 1036L; 1150D and 1201D), whether wild type or heterozygous for an MC1R variant allele (e.g. 1150D and 1201D, respectively) encountered comparable levels of CPD.

**Effects of MC, EC and MC1R genotype on CPD repair**

We compared the rate of repair of CPD in neonatal melanocytes irradiated with 21 mJ/cm² UVR by monitoring the rate of reduction in CPD levels 12, 24, and 36 h post irradiation. Eight neonatal melanocyte cultures were compared, six of which expressed functional MC1R, and consisted of two cultures with low (1207L and 1292L), two cultures with intermediate
(1150D and 1186D), and two cultures (1217D$^+$ and 1293D$^+$) with high MC and EC. The remaining 2 cultures, 830L and 849D, expressed loss-of-function MC1R, with the former having low, and the latter having intermediate MC and EC, respectively. We did not find a correlation between MC or EC and the rate of CPD removal, as determined by multivariate regression analysis (p=0.1215 for the 12 h data point, and p=0.4535 for the 24 h data point). However, we found that melanocytes with loss-of-function MC1R either failed to repair CPD, or had markedly slower repair kinetics compared to all other melanocyte cultures tested, suggesting that MC1R is involved in the regulation of NER (Fig. 5).

**Influence of MC and EC on the release of hydrogen peroxide from UVR-irradiated melanocytes**

Irradiation of human melanocytes with increasing doses of UVR resulted in dose-dependent release of hydrogen peroxide. Melanocytes with high MC and EC released significantly more hydrogen peroxide than their counterparts with low MC and EC (Fig. 6). In the experiment presented in Fig. 6, we found that melanocytes with high MC and EC released 4-fold more hydrogen peroxide after irradiation with 21 mJ/cm$^2$ UVR, and twice the amount of hydrogen peroxide following exposure to 35 mJ/cm$^2$ UVR, than melanocytes with low MC and EC following irradiation with 21 or 35 mJ/cm$^2$ UVR, respectively. Similar results were obtained with adult melanocytes with high, intermediate or low MC and EC (data not shown), suggesting that high MC and EC predisposes melanocytes to release more hydrogen peroxide, possibly due to increased generation of reactive oxygen species during melanin synthesis (Mastore, 2005).
Discussion

The goal of this study was to investigate the effects of melanin content and MCIR genotype on UVR-induced cytotoxicity, major DNA damage, and reactive oxygen species generation in cultured human melanocytes. Using a panel of 19 neonatal and 3 adult human melanocyte cultures, we measured the dose-dependent effects of UVR on their proliferation and survival, induction and repair of CPD, and release of hydrogen peroxide. We correlated the responses of these cultures with their respective MC and EC, and MCIR genotype and ability to respond to α-MSH.

Analysis of MC and EC of the selected panel of melanocyte cultures allowed us to categorize them into three groups, as previously described (Wakamatsu et al., 2005) (Table 1). Among the neonatal melanocyte cultures, the group designated as (L) represented the lightest melanocyte cultures with MC values between 1 and 10 µg/10⁶ cells, and EC values between 0.66 and 4.13 µg/10⁶ cells; (D) represented the intermediately pigmented cultures with MC values between 16.72 and 67.42 µg/10⁶ cells, and EC values between 10.64 and 56 µg/10⁶ cells; and (D⁺) represented the darkest cultures, with the highest MC and EC values, ranging between 82.83 and 142 µg/10⁶ cells, and 76.16 and 100 µg/10⁶ cells, respectively. The chemical analysis of EC revealed that it correlated directly and consistently with MC and the visual pigmentation of the various cultures, as assessed by light microscopic examination of the cultures and the color of the skin of origin. On the other hand, pheomelanin contents varied and did not necessarily correlate with MC. The same observations were made in the adult melanocyte cultures, in which MC and EC precisely reflected the ethnic background of the donors, consistent with the results reported in recent studies conducted on skin biopsies from donors with different ethnic origins (Tadokoro et al., 2003), (Hennessy et al., 2005), and with a larger study that we have finished on
cultured human melanocytes (Wakamatsu et al., 2005). Therefore, we conclude that EC is the main contributor to the diversity of cutaneous pigmentation. This quantitative analysis of melanins provided an objective assessment of the pigmentary status of melanocytes, and allowed for more precise comparison of the responses of melanocytes, with either similar or different pigmentary profiles, to UVR.

As expected, exposure of melanocytes to increasing doses of UVR resulted in dose-dependent growth arrest and apoptosis, both of which were more pronounced in melanocytes with lower rather than higher MC and EC (Fig. 1, 2). Previously, we reported that UVR reduced the proliferation of melanocytes by arresting them in G1, and induced apoptosis (Barker et al., 1995); (Kadekaro et al., 2003), (Kadekaro et al., 2005). We regard apoptosis as outcome of extensive DNA damage that surpasses cellular DNA repair capacity. While melanocytes in the skin are resistant to apoptosis, they undergo apoptosis in vitro in the absence of paracrine survival factors, such as NGF, endothelin-1 or α-MSH in the culture medium (Zhai et al., 1993); (Kadekaro et al., 2005). Annexin V staining of UVR-irradiated melanocytes confirmed the results presented in Fig. 1C showing that in melanocytes expressing functional MC1R, those with low MC and EC encountered more extensive apoptosis than their counterparts with high MC and EC (Fig. 2). These results argue for the importance of EC in reducing the cytotoxic effect of UVR on melanocytes, possibly by acting as a shield that limits the extent of UVR exposure, and as a scavenger of reactive oxygen species (Pathak et al., 1971), (Kobayashi et al., 1998), (Bustamante et al., 1993).

The protective effect of EC against UVR-induced cytotoxicity was evident in neonatal as well as in adult melanocytes, but was largely contingent on the expression of functional MC1R. Previously, we reported that melanocytes with loss-of-function MC1R due to expression of
certain variants of the \textit{MC1R} gene, namely R151C, R160W, and D294H, in the homozygous or compound heterozygous state, are more prone to UVR-induced cytotoxicity than their counterparts with functional MC1R (Scott et al., 2002a). Recently, we showed that the ability of human melanocytes to respond to melanocortins promotes their survival after exposure to UVR (Kadekaro et al., 2005). Here, we obtained similar results, and we found that the extent of apoptosis after exposure to doses equal to or greater than 21 mJ/cm$^2$ UVR was greater in melanocytes that were non-responsive to $\alpha$-MSH than their counterparts that were responsive to melanocortins (Fig. 2).

We found that irradiation of melanocytes with 7, 14, 21, or 35 mJ/cm$^2$ UVR reduced the levels of Bcl2 dose-dependently (Fig. 3B). The observed reduction in Bcl2 levels further confirms that UVR-induced cell killing occurs by apoptosis, as suggested by the results of Annexin V staining (Fig. 2). Bcl2 is expressed constitutively in human melanocytes and is crucial for melanocyte survival (Plettenberg et al., 1995); (Veis et al., 1993). The observations that the survival effects of NGF, endothelin-1 and $\alpha$-MSH on UVR-irradiated melanocytes involve up regulation of Bcl2 levels further underscore the importance of Bcl2 in melanocyte survival (Zhai et al., 1993), (Kadekaro et al., 2005).

Exposure of human melanocytes to UVR results in the induction of DNA photoproducts, the major form of which is CPD (Barker et al., 1995), (Mitchell et al., 1991). Comparison of the induction of CPD in various neonatal human melanocyte cultures demonstrated an inverse correlation between MC and EC values, and CPD levels, as shown through regression analysis (Fig. 4A and B). Lightly pigmented melanocytes (L) sustained high levels of CPD, while those with the highest MC and EC (D$^+$) had the least induction of CPD (Fig. 4A and B). These results support the photoprotective role of EC, which was also evident in adult melanocytes (Fig. 4C).
A recent report comparing the levels of DNA photoproducts in individuals from different ethnic backgrounds and pigmentary phenotypes found that induction of photoproducts was least in the skin of African Americans, followed by Hispanic-Latinos, then Asians, and was highest in white Caucasians (Tadokoro et al., 2003). The similarity between these results obtained from skin that was irradiated in situ, and our results derived from UVR-irradiated cultured human melanocytes validate the use of this in vitro model for investigating the differences in the photobiological responses of individuals with different pigmented phenotypes.

Remarkably, the role of EC in limiting CPD induction is markedly compromised in melanocytes expressing loss-of-function MC1R, which incurred the highest levels of CPD. One of these cultures, 849D, had intermediate EC (14.9 µg/10^6 cells), yet the levels of CPD incurred were higher than those of melanocytes with very low EC, such as in culture 1292L, which had EC of 4.13 µg/10^6 cells. Our results are in agreement with those of previous epidemiological data showing that the MC1R determines the extent of UVR-induced damage and melanoma risk, independently of skin or hair color (Palmer et al., 2000), (Kennedy et al., 2001), (Matichard et al., 2004), (Landi, 2005). Although loss-of-function MC1R alleles are strongly associated with red hair, fair skin, and low EC, these alleles are necessary but not sufficient for this phenotype, and are also expressed in some individuals with olive skin color. This strongly suggests that the MC1R genotype affects the response of cultured melanocytes to UVR, independently of EC or MC.

It has been suggested that MC1R variants are not truly recessive, and that they have a heterozygous effect (Palmer et al., 2000), (Kennedy et al., 2001). Here, we found that human melanocyte cultures heterozygous for a melanoma-associated allele (e.g. 1201D, heterozygous for D294H, and 1292L, heterozygous for V60L) which responded vigorously to α-MSH with a
dose-dependent increase in tyrosinase activity and proliferation (data not shown), did not sustain higher levels of CPD than their counterparts (1150D) that expressed wild type MC1R, and comparable EC and MC (Fig. 4A). Additional melanocyte cultures are currently being tested to further confirm these results. To address the question whether MC of the skin affects the rate of repair of DNA photoproducts, we compared the rate of repair of CPD induced by exposure to 21 mJ/cm² UVR in a panel of melanocyte cultures (Fig. 5). Our results showed no correlation between EC and the rate of CPD removal, consistent with a previous study carried out on individuals with different ethnic backgrounds (Tadokoro et al., 2003) and an earlier study using cultured human melanocytes derived from skin types I or VI (de Leeuw et al., 2001). We found that the cultures with loss-of-function MC1R had the least DNA repair, regardless of their EC (Fig. 5). Direct comparison of CPD repair rate in these cultures and in cultures with comparable EC but functional MC1R revealed striking differences, as the latter were more proficient in repairing their DNA (Fig. 5 insert). Our present findings are consistent with our earlier results showing that melanocortins enhance the repair of CPD in melanocytes, an effect that requires functional MC1R (Kadekaro et al., 2005). In the experiments hereby presented, bovine pituitary extract, supplemented in the culture medium, is the source of melanocortins.

Exposure to UVR also results in the generation of reactive oxygen species that can cause lipid peroxidation, protein degradation, and oxidative DNA damage (Sander et al., 2004). Recently, we reported that irradiation of human melanocytes with UVR increased the release of hydrogen peroxide (Kadekaro et al., 2005) and the formation of 8-hydroxy deoxyguanosine residues, a major form of oxidative DNA damage in melanocytes (unpublished results). Here, we compared the levels of hydrogen peroxide released by melanocytes with different MC and EC following irradiation with increasing doses of UVR (Fig. 6A and B). We observed a dose-
dependent increase in the release of hydrogen peroxide, which was greater in melanocytes with high MC and EC than in melanocytes with low MC and EC, mainly in the groups irradiated with 21 or 35 mJ/cm² UVR (Fig. 6 A and B). Similar differences were observed in 3 adult melanocyte cultures with high, intermediate, and low EC, respectively (data not shown). Since dark melanocytes have a high melanogenic activity, we postulate that the high levels of hydrogen peroxide are due to its increased synthesis during the oxidation of DOPA (Mastore, 2005).

Comparison of CPD induction, hydrogen peroxide release, and apoptosis in melanocyte cultures with different MC and EC revealed that CPD induction alone does not totally account for the UVR-induced apoptosis. After exposure to 7 mJ/cm² UVR, L melanocytes encountered high levels of CPD, very low levels of hydrogen peroxide, and did not undergo apoptosis (Table 1, Fig. 2, 4, and 6). However, D⁺ melanocytes encountered low levels of CPD, very high levels of hydrogen peroxide, and underwent apoptosis in response to 35 mJ/cm² UVR. These results implicate the generation of reactive oxygen species that can damage DNA, proteins and lipids, in melanocyte apoptosis. As melanocortins reduce the release of hydrogen peroxide from UVR-irradiated melanocytes, we expect melanocytes that are refractory to melanocortins to have reduced antioxidant defenses, and to sustain more oxidative stress than melanocytes that respond to melanocortins (Kadekaro et al., 2005).

In summary, the data hereby presented show that while EC is an important factor that affects the extent of UVR-induced DNA damage and cytotoxicity of human melanocytes, its photoprotective role is compromised by the absence of functional MC1R and inability to respond to melanocortins. Our results provide experimental support for epidemiological data that found melanoma risk correlates inversely with skin pigmentation. However, the photoprotective effect of melanin, particularly eumelanin, is diminished in individuals with olive skin color that express
loss-of-function \textit{MCIR} (Palmer et al., 2000); (Kennedy et al., 2001). Given the importance of repair of UVR-induced DNA damage in melanoma prevention, the diminished ability of melanocytes with loss-of-function \textit{MCIR} to repair DNA efficiently offers an explanation for the role of the \textit{MCIR} gene as a melanoma susceptibility gene.

\textbf{Methods}

\textbf{Melanocyte Culture.} Primary human melanocyte cultures were established from 19 different foreskins from anonymous newborns, and 3 adult skin biopsies obtained after informed consent, and maintained under identical culture conditions as described (Abdel-Malek et al., 1995). Cultures were classified as L, for very light, D for intermediate-dark, and D+ for very dark, based on visual pigmentation, as depicted by light microscopy and the color of the skin of origin. Low passage cultures (<10) were used for all experiments to insure minimal genetic drift \textit{in vitro}.

\textbf{Analysis of eumelanin and pheomelanin contents and \textit{MCIR} genotype.} For analysis of eumelanin and pheomelanin contents, melanocytes were harvested, counted, pelleted and lyophilized before being analyzed using a microassay developed by Ito and Fujita (Ito and Fujita, 1985). Each lyophilized sample, containing approximately 1x10^6 melanocytes was oxidized by permanganate to pyrrole 2,3,5-tricarboxylic acid (PTCA) and analyzed by HPLC with ultraviolet detection to determine eumelanin content. To determine pheomelanin content, identical samples were hydrolyzed with hydriodic acid to aminohydroxyphenylalanine (AHP), and analyzed by HPLC with electrochemical detection (Wakamatsu K, 2002). Eumelanin assays were performed in duplicate while pheomelanin assays were as single measurements. Variations of PTCA and AHP values are approximately 10% or less when determined on separate occasions. One ng of PTCA corresponds to 160 ng of eumelanin (Ozeki et al., 1996) while one ng of 4-AHP corresponds to 9 ng of pheomelanin (Wakamatsu K, 2002).
**Sequencing of MC1R gene.** Total RNA was isolated from cultured human melanocytes using the RNA Easy Kit (Qiagen, Valencia, CA) and cDNA was obtained by reverse transcription of 100 ng of total RNA using random hexamers as primers (50 uM Oligo(dT)/20 µl final volume). An equivalent volume of 2 µl of cDNA suspension was used for RT-PCR amplification using SuperScript TMIII, Invitrogen Life Technologies. The entire coding region of the MC1R was amplified in two separate reactions using the following sets of primers: for the first half of the sequence N-terminal primer (5'-GCAGCACCATGAACTAAGCA-3') and C-terminal primer (5'-CCAGCATAGCCAGGAAGAAG-3') and for the second half of the sequence N-terminal primer (5'-GTGGACCGCCTACATCTCCAT-3') and C-terminal primer (5'-GGACCAGGGAGGTAAGGAAC -3'). A PCR touchdown cycling profile was used, consisting of one cycle of 95°C for 5 minutes, followed by 25 cycles of 94°C for 1 minute, 62°C for 1 minute with a 0.5°C/cycle decrease, and 72°C for 1 minute; followed by 10 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute, and a final extension at 72°C for 10 minutes. The PCR products were purified with a QIAquic PCR Purification Kit (QIAGEN Science, Maryland, USA) according to the manufacturer’s instructions and sequenced with an automated system Perkin Elmer/Applied Biosystems instrument models 373A or 377).

**Growth Curve of Melanocytes after Irradiation with UVR.** Neonatal human melanocytes were plated onto 60 mm dishes at a density of 2.5 x 10^5 cells/dish. Two days after plating, cells were washed (2x) and irradiated in phosphate buffer saline (PBS) with a single dose of 7, 14, 21 or 35 mJ/cm² UV emitted by a bank of FS-20 fluorescent UVR lamps that have a continuous emission spectrum with a peak at 313 nm (National Biological, Twinsburg, OH), as described (Barker et al., 1995; Im et al., 1998; Tada et al., 1998). Percent of cell death was determined on days 2, 4 and 6 days after irradiation with UVR by calculating the number of dead melanocytes.
that detached and incorporated Trypan Blue dye, as described (Barker et al., 1995). The cell number in each dish (triplicate dishes in each group) was counted using a Coulter Counter (model ZM).

**Determination of Melanocyte Apoptosis by Annexin V Staining.** Melanocytes were plated and irradiated with UVR as described above. Twenty-four hours after exposure to UVR, melanocytes were harvested and stained with APC-Annexin (BD Pharmingen, San Diego, CA) and propidium iodide (Sigma Chemical Co., St. Louis, MO). Samples were analyzed by flow cytometry immediately after staining on BD LSR, and the data were analyzed by CELLQuest software.

**Expression of Bcl2 after irradiation with UVR.** Human melanocytes were plated onto 100 mm dishes at a density of 1.5 x 10^6 cells/dish. Cells were irradiated as described above and cell extracts prepared 24h after UVR exposure, using RIPA buffer containing a cocktail of protease inhibitors. Fifteen to 20 µg protein/group were separated on 12% gel, and transferred onto nitrocellulose membranes (BioRad). Western blot analysis was carried out using Bcl2 Ab-1 monoclonal antibody (Oncogene Research Poducts). The membrane was then incubated with horseradish peroxidase-conjugated anti-mouse IgG (Calbiochem, La Jolla, CA). Membranes were also reacted with antibody for actin-C-11 horseradish peroxidase IgG (Santa Cruz Biotechnology, Santa Cruz, CA) to control for loading. The respective bands were visualized using Enhanced Chemiluminescence (Amersham, Arlington Heights, IL).

**Southwestern Blot Analysis of CPD.** Melanocytes were plated onto 100 mm dish and irradiated with 0, 7, 14, 21 or 35 mJ/cm². Cells were harvested immediately after UVR exposure (0 h) to determine induction of CPD. The dose of 21 mJ/cm² UVR was selected to determine the
repair of CPD since it induced a highly significant amount of CPD compared to controls (p=0.0094), but did not kill the majority of cultured melanocytes. Melanocytes were plated as described, then irradiated with 21 mJ/cm², and harvested immediately (0 h) and 12, 24 and 36 h after irradiation. Genomic DNA was isolated, and Southwestern blot analysis was carried out with triplicate lanes (0.5 µg DNA/lane) loaded for each group, and using monoclonal antibody TDM2 directed against CPD (a gift from Toshi Mori, Nara University, Japan), as described (Kadekaro et al., 2005). The resulting bands were quantified by densitometry, using Alpha Innotech Imaging System and the AlphaEase FC StandAlone Software (San Leandro, CA). Statistical analysis of the induction data was performed by linear regression with SAS Software v9.1.3 (SAS Institute, Inc., Cary, NC). This analysis compared the influence of eumelanin content (x) on CPD formation (y) at each UVR dose. The data are given in Table 2. The normality assumption is breaking down for a simple linear regression model, \( y = \alpha + \beta x + \text{error} \), fit. Box-Cox transformation of the type \( y^\lambda \) for an optimal \( \lambda \) is used to achieve normality (Srivastava, 2002). For the transformed \( y^\lambda \), a simple regression model \( y^\lambda = \alpha + \beta x + \text{error} \) is fitted at each UVR dose (PROC TRANSREG). The estimate of \( \beta \) was significant at each dose. The regression lines are converted to the original units of y and are presented in Figure 4A. Multivariate regression analysis was performed for the repair data and showed no correlation between EC and rate of repair (PROC GLM).
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References


Table 1. Melanin contents, MCIIR genotypes and responsiveness to α-MSH of melanocyte cultures used in this study

<table>
<thead>
<tr>
<th>Melanocyte Culture</th>
<th>Total Melanin (µg/10^6 cells)</th>
<th>Eumelanin (µg/10^6 cells)</th>
<th>Pheomelanin (µg/10^6 cells)</th>
<th>MC1R genotype</th>
<th>Response to αMSH</th>
</tr>
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<tbody>
<tr>
<td>753L*</td>
<td>1.09</td>
<td>0.66</td>
<td>0.43</td>
<td>R160W/R160W</td>
<td>-</td>
</tr>
<tr>
<td>1036L</td>
<td>8.98</td>
<td>3.43</td>
<td>5.55</td>
<td>R160W/+</td>
<td>±</td>
</tr>
<tr>
<td>830L*</td>
<td>5.26</td>
<td>3.46</td>
<td>1.80</td>
<td>R160W/D294H</td>
<td>-</td>
</tr>
<tr>
<td>1257L*</td>
<td>6.06</td>
<td>1.86</td>
<td>4.20</td>
<td>WT</td>
<td>-</td>
</tr>
<tr>
<td>849D*</td>
<td>16.72</td>
<td>14.90</td>
<td>1.82</td>
<td>R151C/D294H</td>
<td>-</td>
</tr>
<tr>
<td>1282L</td>
<td>5.12</td>
<td>2.34</td>
<td>2.78</td>
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<td>+</td>
</tr>
<tr>
<td>1207L</td>
<td>6.42</td>
<td>2.56</td>
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<td>WT</td>
<td>+</td>
</tr>
<tr>
<td>1300L</td>
<td>7.22</td>
<td>3.74</td>
<td>3.47</td>
<td>N.D.</td>
<td>+</td>
</tr>
<tr>
<td>1292L</td>
<td>10.07</td>
<td>4.13</td>
<td>5.94</td>
<td>V60L/+</td>
<td>+</td>
</tr>
<tr>
<td>1186D</td>
<td>17.25</td>
<td>10.64</td>
<td>6.61</td>
<td>N.D.</td>
<td>+</td>
</tr>
<tr>
<td>1150D</td>
<td>46.57</td>
<td>32.96</td>
<td>13.61</td>
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</tr>
<tr>
<td>1191D</td>
<td>48.16</td>
<td>41.60</td>
<td>6.56</td>
<td>N.D.</td>
<td>+</td>
</tr>
<tr>
<td>1201D</td>
<td>53.45</td>
<td>33.84</td>
<td>19.61</td>
<td>D294H/+</td>
<td>+</td>
</tr>
<tr>
<td>1217D</td>
<td>67.42</td>
<td>56.00</td>
<td>11.42</td>
<td>N.D.</td>
<td>+</td>
</tr>
<tr>
<td>1170D</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>+</td>
</tr>
<tr>
<td>1306D+</td>
<td>82.83</td>
<td>76.16</td>
<td>6.67</td>
<td>N.D.</td>
<td>+</td>
</tr>
<tr>
<td>1290D+</td>
<td>94.57</td>
<td>81.76</td>
<td>12.81</td>
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<td>+</td>
</tr>
<tr>
<td>1293D+</td>
<td>128.66</td>
<td>98.08</td>
<td>30.58</td>
<td>N.D.</td>
<td>+</td>
</tr>
<tr>
<td>1272D+</td>
<td>141.42</td>
<td>100.00</td>
<td>41.42</td>
<td>N.D.</td>
<td>+</td>
</tr>
<tr>
<td>AHML</td>
<td>51.17</td>
<td>9.92</td>
<td>41.25</td>
<td>WT</td>
<td>+</td>
</tr>
<tr>
<td>SBD</td>
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<td>77.28</td>
<td>21.59</td>
<td>WT</td>
<td>+</td>
</tr>
<tr>
<td>AHMD+</td>
<td>345.70</td>
<td>313.44</td>
<td>32.26</td>
<td>N.D.</td>
<td>+</td>
</tr>
</tbody>
</table>

WT = wild type; N.D. = not determined; L = lightly pigmented; D = intermediate pigmented; D+ = darkly pigmented
Figure 1A

Figure 1B

Figure 1C
Figure 2

![Figure 2 - Bar graph showing % increase in apoptosis with varying UVR doses (mJ/cm²) for different cell lines.]

Figure 3

![Figure 3 - Western blot showing Bcl2 and Actin protein expression with varying UVR doses (mJ/cm²).]