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Cutaneous photoprotection and melanoma susceptibility: reaching beyond melanin content to the frontiers oF DNA repair

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1. ABSTRACT

Melanoma is the most devastating form of skin cancer. The steady increase in the incidence of melanoma, its resistance to chemotherapy, together with its high potential to metastasize, have emphasized the importance of its prevention. It is becoming clear that solar ultraviolet radiation is a main culprit in the etiology of melanoma, the same as in basal and squamous cell carcinomas. It is commonly accepted that skin pigmentation and melanin content are principal determinants of the susceptibility to melanoma and other sun-induced skin cancers. Although this is generally true, however, prediction of melanoma risk based solely on pigmentary phenotype is not always precise and fails to identify high-risk individuals with dark skin color. Other important risk factors need to be considered and better defined, particularly DNA repair capacity. Emerging studies have revealed the role of melanoma susceptibility genes in regulating DNA repair, and indicated that melanoma patients have a lower DNA repair capacity than the general population. As the response of human melanocytes to ultraviolet radiation is modulated by an array of paracrine factors, we have focused our investigation on the role of melanocortins and the melanocortin 1 receptor, as well as endothelin-1, in this response. We have discovered novel roles for melanocortins and endothelin-1 as survival factors that rescue human melanocytes from ultraviolet radiation-induced apoptosis, and importantly enhance repair of DNA photoproducts and reduce the release of hydrogen peroxide that can cause oxidative stress. Our findings, together with epidemiological data showing that loss-of-function mutations in the melanocortin-1 receptor gene increase the risk of melanoma, substantiate the role of DNA repair in melanoma genesis, and suggest that responsiveness to melanocortins and endothelin-1 is important for melanoma prevention.

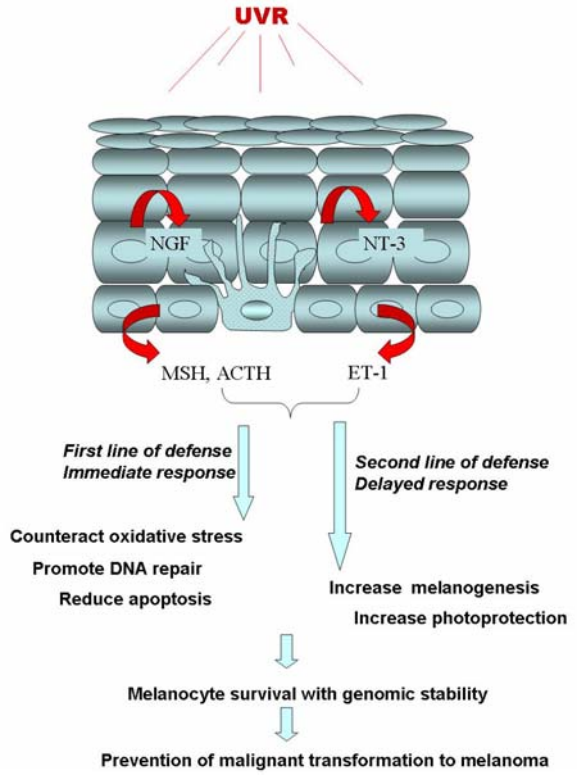

Figure 1. Proposed role of UVR-induced paracrine factors, particularly melanocortins and ET-1, in the response of human melanocytes to UVR. Exposure of the skin to UVR activates a paracrine network in the skin by increasing the synthesis of a variety of growth factors and cytokines, and/or their respective receptors. Some of those factors, such as NGF, NT-3, MSH and ACTH, and ET-1, function as survival factors that inhibit UVR-induced apoptosis of melanocytes. We have shown that in addition to promoting melanocyte survival, MSH and ACTH, and ET-1, also enhance the repair of CPD and reduce the release of hydrogen peroxide, thus reducing oxidative stress. These effects represent the immediate response to UVR, which is then followed by the delayed response, which involves increased melanogenesis, and thus photoprotection. The effects of melanocortins and ET-1 are expected to maintain genomic stability of melanocytes and reduce the chance for malignant transformation to melanoma.

2. INTRODUCTION

In the cutaneous epidermis, melanocytes constitute a minor cell population that is specialized in the synthesis of melanin, the pigment that gives the skin its distinctive color. Malignant transformation of melanocytes results in melanoma, the most fatal form of skin cancer. To date, other than surgical resection, no effective chemo- or radiotherapeutic strategy exists for eradication of melanoma. Melanoma tumors generally have a high metastatic potential, and most patients succumb to metastatic disease. The resistance of melanoma tumors to various forms of chemotherapy has emphasized the importance of prevention and early detection. The development of effective strategies for melanoma prevention requires the elucidation of the factors that determine melanoma susceptibility and the mechanism(s) of melanocyte transformation . Cutaneous pigmentation is regarded as the main photoprotective mechanism against sun-induced skin cancer, including melanoma (1-2). Skin pigmentation is the outcome of melanin synthesis by epidermal melanocytes and the transfer of melanin containing organelles, melanosomes, from melanocytes to surrounding keratinocytes (3). The significance of pigmentation in skin cancer prevention is based on epidemiological and clinical data demonstrating that the incidence of melanoma and non-melanoma skin cancer is highest in individuals with fair skin who burn rather than tan when exposed to the sun [reviewed by (4), (5-6)]

In addition to pigmentary phenotype, there are other factors that determine melanoma susceptibility. Data from various laboratories emphasized the significance of DNA repair capacity in determining the risk of melanoma (7-9). Recent data from our laboratory revealed that the melanocortins alpha-melanocyte stimulating hormone (MSH) and adrenocorticotropic hormone (ACTH), as well as endothelin-1 (ET-1), known to regulate human melanocyte proliferation and melanogenesis, are survival factors that rescue human melanocytes from apoptosis induced by ultraviolet radiation (UVR;10). Importantly, these survival effects are accompanied by increased repair of DNA photoproducts and reduced release of hydrogen peroxide that can cause oxidative stress from UVR-irradiated human melanocytes. All of these effects precede the increase in melanin content. These intriguing findings suggest the following paradigm for the role of the paracrine factors MSH, ACTH and ET-1in the response of melanocytes to UVR (figure 1). These three factors activate survival, DNA repair, and antioxidant pathways in human melanocytes, thus are expected to prevent apoptosis and maintain genomic stability. The pigmentary effects of melanocortins and ET-1 proceed as a delayed response and protect against the damaging effects of subsequent UVR exposures. Epidermal melanocytes, for the most part, are a population of highly differentiated cells that rarely proliferate and have a low self-renewal capacity. In the skin, melanocytes persist for decades, and this longevity might allow ample time for accumulation of mutations that might ultimately result in malignant transformation to melanoma. Given the importance of the melanocyte in photoprotection of the skin, insuring its survival and genomic stability is critical for prevention of UVR-induced skin cancer. In addition to the melanocortins and ET-1, there are other paracrine factors, such as nerve growth factor (NGF), neurotrophin-3 (NT-3), and mast cell growth factor (MGF) that promote human melanocyte survival, yet their effects on DNA repair are unknown (11-13).

The effects of MSH on human melanocytes are mediated by binding and activating the melanocortin 1 receptor (MC1R) (14-15). The *MC1R* gene is highly polymorphic, and some of its alleles are strongly associated with red hair phenotype, poor tanning ability and increased risk of melanoma (16-26). Our investigation of some of these alleles in cultured human melanocytes revealed that

they represent loss-of-function mutations in the *MC1R* gene (15). Our results were corroborated by pharmacological studies on the different *MC1R* variants (27). Human melanocytes that express these alleles have a reduced capacity to repair UVR-induced DNA photoproducts and to counteract oxidative stress (10). Thus, we conclude that inability of such melanocytes to respond to MSH impairs their DNA repair capacity and antioxidant defenses, and renders them more vulnerable to UVR-induced mutagenesis and malignant transformation.

In this review, we will discuss the current knowledge about the photoprotective role of pigmentation, the role of UVR in the etiology of melanoma, the signaling pathway for UVR in human melanocytes, and how the response to melanocortins and ET-1 affects the susceptibility to melanoma.

3. PHOTOPROTECTIVE ROLE OF MELANIN

The function of epidermal melanocytes is to synthesize the pigment melanin, which is considered the principal photoprotective agent against the deleterious effects of solar UVR on the skin, the most drastic of which is skin cancer (1-2, 5-6, 28-29). Within the melanocyte, melanin is synthesized in melanosomes, which are transferred to surrounding keratinocytes, where they form supranuclear caps that protect nuclear DNA from impinging UV rays (3, 30, 31). Melanin has been shown to act as a shield that reduces the penetration of short and long wavelength UVR through the epidermal layers (1, 32). More UV rays penetrate through light skin than dark skin, and as a consequence, the extent of UVR-induced DNA photoproducts is substantially higher in the former than in the latter skin (32-33). Comparison of the extent of DNA photoproducts induced by 1 minimal erythemal dose (MED) of UVR in the skin of individuals from different ethnic backgrounds revealed that the amount of DNA damage correlated inversely with constitutive melanin content (33). In addition to its shielding effect, melanin also scavenges reactive oxygen species, thus reducing oxidative damage to proteins, lipids and DNA (34-35).

Further support for the photoprotective role of melanin comes from extensive epidemiological data documenting that the incidence of sun-induced skin cancers is remarkably higher in individuals with fair skin and red hair, who burn rather than tan after sun exposure, than in those with dark skin who tan efficiently when exposed to the sun (4-6, 36). Inability of melanocytes to synthesize melanin, as in albinism, increases the risk for skin cancer (37). In general, an inverse relationship exists between skin pigmentation and the incidence of skin cancer, including melanoma. In the U.S. A., the risk for melanoma is 10 times lower in African Americans than in Caucasians, and in Australia, the highest incidence of melanoma worldwide is in the Celtic population (38-39).

Generally, constitutive pigmentation determines the tanning ability of the skin after sun exposure. This association has led to the classification of pigmentary phenotypes into 6 phototypes, skin types I-VI, with skin type I being the least pigmented and having the least tanning ability, and skin type VI being the darkest and having the most tanning ability (3). Skin type I individuals are the ones with the highest risk for skin cancer. This classification, however, is subjective, and there is an urgent need for more objective and quantitative classification of skin pigmentary phenotypes, such as that based on melanin content, and MED that correlates directly with constitutive pigmentation (33, 40).

That melanogenesis is induced by UVR, and also by inflammation that results in postinflammatory hyperpigmentation, suggests that increased melanin production is a stress response, indicative of DNA damage or DNA repair. Introduction of oligonucleotides in the form of pT-pT dimers or telomere overhang into melanocytes was found to activate nucleotide excision repair and melanogenesis (41). Also, repetitive exposures to small doses of UVR were found to increase DNA repair in skin type IV individuals who tan, but not in skin type II individuals with a poor tanning ability, suggesting that DNA repair capacity correlates with tanning ability (42). Importantly, this concept links melanogenesis to DNA repair and might explain the difference in skin cancer risk among individuals with different skin phototypes. This concept also suggests that individuals with different skin phototypes have different DNA repair capacities.

4. REGULATION OF SKIN PIGMENTATION

4.1. Regulation of eumelanin and pheomelanin synthesis

Human pigmentation is a polygenic trait that is determined by more than 100 genes. The tremendous diversity of human pigmentation is mainly attributed to the wide variation in melanin contents. Epidermal melanocytes synthesize the two major types of melanin, the black-brown eumelanin, and the red-yellow pheomelanin (43, 44). The former is superior in its photoprotective properties, mainly due to its resistance to degradation by UVR and its higher efficiency in quenching oxygen radicals (45). Total melanin content and the levels of eumelanin correlate directly with the extent of skin pigmentation in vivo (44, 46). In cultured human melanocytes, eumelanin content consistently correlates directly with total melanin content, as well as with the visual pigmentation of the melanocytes and the pigmentary phenotype of the skin from which they were derived (47). Melanocytes cultured from lightly pigmented Caucasian skin have the least, while those cultured from Asian-Indian donors have higher, and those derived from African American donors have the highest total melanin and eumelanin contents (figure 2). Melanocytes cultured from lightly pigmented human skin express lower basal activity of tyrosinase, the rate-limiting enzyme in the melanogenic pathway, as well as lower protein levels of tyrosinase, and tyrosinase related proteins (Tyrp)-1 and -2 than melanocytes cultured from dark skin (48-50). It is thought that tyrosinase activity is subject to regulation by melanosomal pH, which is acidic in lightly pigmented melanocytes, and neutral in dark melanocytes (51-52).

The last decade of the twentieth century witnessed remarkable progress in the understanding of the

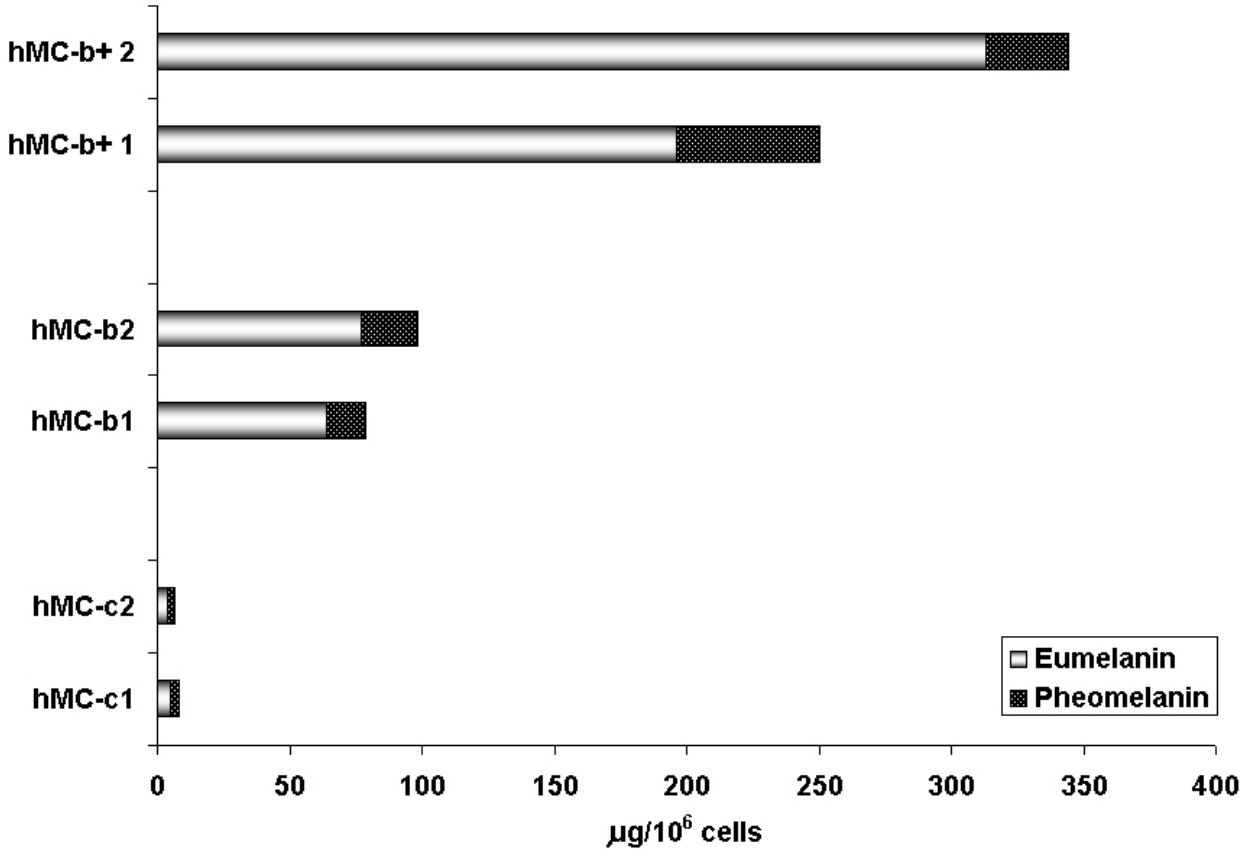

Figure 2. Comparison of eumelanin and pheomelanin contents in the skin of individuals from different ethnic groups and with different pigmentary phenotypes. Eumelanin and pheomelanin contents were determined by HPLC in cultured melanocytes derived from two lightly pigmented Caucasian donors (hMC-C1 and hMC-C2), two Indian donors (HMC-b1 and HMC-b2), and two African American donors (hMC-b+1, and hMC-b+2). The highest eumelanin contents were expressed by melanocytes from African American donors, and the least were expressed by melanocytes from lightly pigmented Caucasians.

genetic and molecular regulation of eumelanin and pheomelanin synthesis in human melanocytes. It became evident that in human melanocytes, similar to mouse melanocytes, eumelanin synthesis is primarily regulated by the *MC1R* gene, the homolog of the *extension* locus in the mouse, and the *proopiomelanocortin (POMC)* gene (53-54, 16-17, 19, 55). The former codes for the receptor of melanocortins that is expressed mainly by human melanocytes, and the latter codes for POMC protein, the precursor peptide of all melanocortins (alpha-, beta-, gamma-, and ACTH), as well as other bioactive peptides, such as beta-lipotropic hormone and beta-endorphin (56-57). Pheomelanin synthesis is mainly regulated by the human agouti gene, which codes for agouti signaling protein (ASP), the physiological antagonist of the MC1R (58-59). We have demonstrated that cultured human melanocytes respond to melanocortins, particularly MSH and ACTH, with increased melanogenesis and proliferation, and that these effects are mediated by activating the MC1R (60, 14). Studies on the mechanism of action of melanocortins on melanocytes revealed that the synthesis of eumelanin requires a high activity of the enzyme tyrosinase and high protein levels of tyrosinase and Tyrp-1 and -2, all of which are increased upon treatment with either MSH or ACTH (60-62). On the other hand, pheomelanin synthesis ensues when the activity of tyrosinase and the expression of tyrosinase, Tyrp-1 and -2 are greatly reduced, as observed following treatment of human melanocytes with ASP (59). These landmark discoveries suggest that the *MC1R* and *agouti* genes play an important role in regulating constitutive pigmentation.

Population studies revealed that the diversity of human pigmentation is due to a large extent to the high polymorphism of the *MC1R* gene, which has more than 45 different variants (17, 19-20). Interestingly, the consensus *MC1R* gene is predominantly expressed in Africans, and thus is associated with high eumelanin content of the skin (63). On the other hand, many *MC1R* variant alleles are prevalent in Northern European countries and Australia, and some variants, particularly R160W, R151C, and D294H substitutions, are strongly associated with red hair phenotype (17,19). The *MC1R* genotype is an important determinant of constitutive, as well as facultative (i.e. UVR-induced) pigmentation. *MC1R* variants that are associated with red hair also reduce the tanning response of

the skin to UVR (64-65). Importantly, the *MC1R* gene determines the risk for melanoma, independently of skin or hair color, since some variants are expressed in individuals with dark skin (21, 23, 25-26).

5. THE ETIOLOGY OF MELANOMA: ROLE OF UVB RADIATION

It is widely accepted that exposure to solar UVR, particularly in the UVB spectrum (290-320 nm wavelength), is the main etiological factor for non-melanoma skin cancer tumors, i.e. squamous and basal cell carcinoma (66-67). "UVB signature" mutations, particularly in the tumor suppressor gene *p53*, occur during the early stages of these tumors (66-68). The scarcity of p53 mutations in melanoma tumors led to skepticism about the importance of UVBR in the etiology of melanoma (69). Based on the findings that UVAR induces melanoma tumor formation in the Xiphophorus fish, it was suggested that exposure to UVAR (320-400 nm) results in melanoma formation (70-71). However, such an effect of UVAR could not be demonstrated in other animal models for melanoma, and recent data suggest that the emission spectrum for melanoma in the HGF transgenic mouse model is in the UVB, not the UVA, range (72). Despite these recent results, contribution of UVAR to skin carcinogenesis should not be totally discounted, since solar UVAR is by far more abundant than UVBR, and can enhance UVBR-induced photocarcinogenesis via the generation of reactive oxygen radicals, as well as induction of DNA photopoducts (2, 73).

There are several lines of evidence that support the significance of UVBR-induced DNA damage in the malignant transformation of human melanocytes. First, patients with the disease xeroderma pigmentosum, characterized by deficient nucleotide excision repair, the main repair pathway for UVBR-induced DNA photoproducts, have 100 fold higher incidence of melanoma, compared to the general population (7). Second, patients with melanoma have a lower capacity to carry out nucleotide excision repair than disease-free individuals (8). Third, mutations in the melanoma susceptibility locus *CDKN2A* that lead to aberrant p16 or Arf expression compromise the capacity to repair DNA photoproducts (9). Fourth, p16 mutations that have been detected in sporadic melanoma tumors are UVB signature mutations (74, 75).

6. RESPONSE OF HUMAN MELANOCYTES TO UVR

Cultured human melanocytes represent a valuable tool to investigate the regulation of human pigmentation and the effects of UVR. Human melanocytes *in vitro* express total melanin and eumelanin contents characteristic of the pigmentary phenotype of the skin from which they were derived [(47, 50); figure 2]. The response of cultured melanocytes to UVR is comparable to the *in vivo* response of the skin from which they were derived. For example, melanocytes cultured from dark skin, but not melanocytes derived from light skin, exhibit an increase in melanin content, simulating the tanning response following exposure to UVR (76). Also, after a single irradiation with equal doses of UVR, melanocytes from dark skin encounter less cyclobutane pyrimidine dimers (CPD) than melanocytes from light skin[(76-78); figure 3]. In addition to induction of DNA photoproducts, exposure to UVR results in the generation of reactive oxygen species, as exemplified by the release of hydrogen peroxide from UVR-irradiated human melanocytes, and induction of oxidative DNA damage in the form of 8-hydroxy-deoxyguanosine (78, 10).

Irradiation of cultured human melanocytes with UVR results in dose-dependent growth arrest and apoptosis (10, 47-76). An immediate effect of UVR is phosphorylation of the MAP kinases p38 and JNK, known to be involved in stress responses, followed by a subsequent increase in the levels of the tumor suppressor p53 (76, 79-82). The UVR-induced growth arrest is mediated by accumulation of p53 in response to DNA damage, increased expression of the p53-dependent cyclin-cdk inhibitor p21, and dephosphorylation of pRb (81, 83). Exposure of cultured human melanocytes to UVR results in decreased level of the anti-apoptotic protein Bcl2, known to be a main regulator of melanocyte survival (10, 84-86). This reduction in Bcl2 level should facilitate apoptosis, which is more pronounced in cultured melanocytes with low melanin content than in melanocytes with high melanin content (our unpublished results).

Exposure of the skin to UVR regulates the synthesis of a wide variety of growth factors and cytokines that function as paracrine or autocrine factors, and modulate the cutaneous response to UVR. Among those factors are the melanocortins MSH and ACTH, as well as ET-1, which regulate human melanocyte proliferation and melanogenesis (14, 60, 86-88). These factors activate signaling pathways that are critical for the melanogenic response to UVR. Endothelin-1 binds and activates the endothelin-B receptor, a G_q protein-coupled receptor with seven transmembrane domains that is the predominant ET receptor expressed on human melanocytes (86, 88). Activation of this receptor results in increased protein kinase C activity and intracellular calcium mobilization, and activates non-receptor tyrosine kinases. The melanocortins MSH and ACTH bind and activate the MC1R, a G_s protein-coupled receptor with seven transmembrane domains (14, 56). Activation of the MC1R results in increased cAMP formation (14-15). The cAMP pathway is regarded as the main signaling pathway for the MC1R, and the principal regulator of melanogenesis (85, 89). In addition to stimulation of melanogenesis, binding of melanocortins to the MC1R enhances dendricity of melanocytes, which is expected to facilitate melanosome transfer to keratinocytes and thus increase pigmentation (60, 90).

In the presence of basic fibroblast growth factor (bFGF), a paracrine mitogen for melanocytes, ET-1 and either MSH or ACTH interact synergistically to stimulate human melanocyte proliferation and melanogenesis (91, 86). Basic FGF and ET-1 activate the MAP kinases ERK1/2, which in turn activate the transcription factor

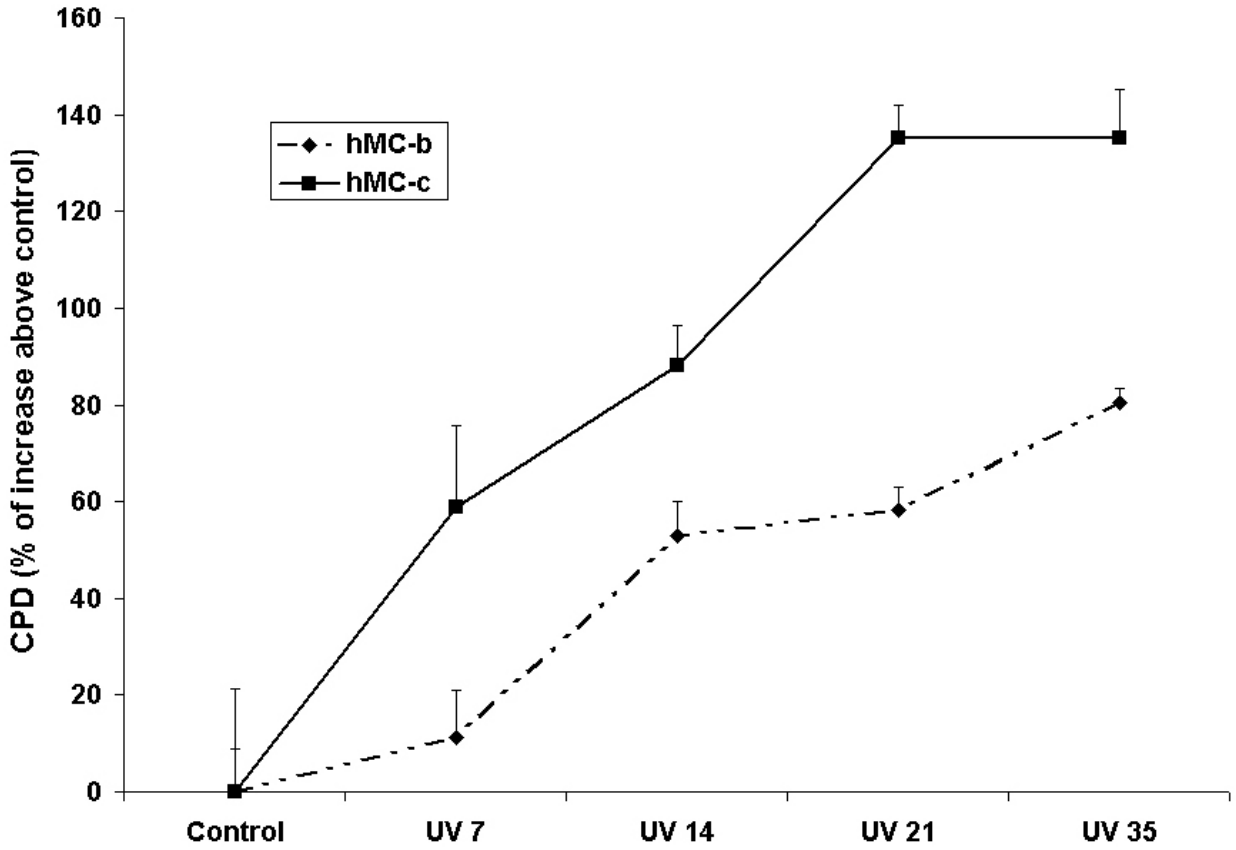

Figure 3. Induction of cyclobutane pyrimidine dimers in light pigmented melanocytes as compared to dark melanocytes. Dose-dependent induction of CPD in melanocytes cultured from lightly pigmented skin (hMC-c), or dark skin (hMC-b) were compared. Melanocytes were irradiated with 7, 14, 21, or 35 mJ/cm^2 from unfiltered FS-20 lamps. DNA was immediately isolated, and CPD formation was determined by Southwestern blot analysis, using a monoclonal antibody against CPD. Each data point represents the average of triplicate lanes ± S.E.

CREB (82). MSH alone does not have these effects, but contributes synergistically to the effects of ET-1 and bFGF on ERK1/2 and CREB phosphorylation. Both MSH and ET-1 increase the protein levels of the phosphorylated, as well as unphosphorylated forms of the transcription factor Mitf, a substrate of CREB (10). An interesting and unexpected finding is that ET-1 and bFGF up regulate the levels of the MC1R mRNA, thus possibly enhancing and/or sustaining the response of melanocytes to melanocortins (92).

We recently performed microarray-based screen to identify the primary target genes of cultured human melanocytes exposed to UVR (unpublished data). Analysis of the data identified genes that were at least 2-fold up or down regulated. As expected, altered gene expression was observed in diverse functional categories such as regulators of cell cycle, DNA repair, growth factors and growth factor receptor, apoptotic and oxidative stress-related genes. Among the down regulated cell cycle genes were Cyclin D1, MYC, RAF1, and MAP kinase 6. Some apoptosis regulatory genes were found to be down regulated, such as SNAI2, BID, caspase 8, TNFAIP1 and 3, and NFκB. As for growth factors and growth factor receptor genes, we found that retinoic acid receptor alpha, and opiod growth factor receptor genes were suppressed. Of particular interest for us, the *MC1R* gene was down regulated 4-8 hours, then up regulated 24 hours after UVR exposure. Among the up regulated genes were thyrotropin releasing hormone (TRH), interleukin 8 receptor, and FGF 17 that we have found to up regulate the levels of the MC1R mRNA (92). Other genes of particular significance for melanocytes, like adenylate cyclase 5 and phospholipase A2, were up regulated, while phospholipase 4B was markedly down regulated. Mitf was initially down regulated, then later up regulated, demonstrating that it is transcriptionally as well as posttranslationally modified in response to UVR. Some important genes that counteract UVR oxidative stress such as glutathione S transferase M1, M5, theta 1, and glutathione peroxidase 3 were up regulated. Also, the expression of GADD45 A, an important DNA repair and cell cycle regulatory gene, was increased. It has been reported that exposure of cultured human melanocytes to simulated solar UVR resulted in increased expression of GADD45 after 4 hours, followed by stimulation of heme oxygenase (HO-1) expression 24 hours after irradiation, as determined by PCR (93). By Western blot analysis, we have detected an increase in the

levels of 1 HO-1 48 hours after UVR exposure, indicative of oxidative stress response, and prolonged expression of the cyclin-cdk inhibitor, p21, beginning as early as 4 hours post irradiation, and lasting for at least 48 hours thereafter (81, 94). Further verification of the microarray results will elucidate the genetic targets of UVR in human melanocytes, and lead to targeted preventative strategies against the genotoxic effects of UVR exposure.

7. MELANOCORTINS AND ENDOTHELIN-1 AS SURVIVAL FACTORS AND ENHANCERS OF DNA REPAIR OF HUMAN MELANOCYTES

Unlike basal or squamous cell carcinoma tumors that arise from keratinocytes, which have a high self-renewal capacity, melanoma tumors arise from melanocytes, most of which are highly differentiated cells with a poor proliferation capacity. Melanocytes have a very long life span and are endowed with mechanisms that enable them to resist apoptsis, such as high expression of the anti-apoptotic protein Bcl2 (95). However, it is predicted that extensive DNA damage that surpasses the DNA repair capacity of melanocytes results in either apoptosis, or survival with mutations due to unrepaired DNA damage. Unlike keratinocytes that undergo a well-characterized differentiation program that ultimately leads to their demise and desquamation, melanocytes persist in human skin for decades (96). This longevity allows ample time for genetic alterations to accumulate. Unless appropriate repair mechanisms are activated, genetic instability prevails and leads to the malignant transformation of melanocytes to melanoma. Given the significance of the melanocyte in photoprotection and its poor ability to self-renew, insuring its survival in the epidermis is critical for prevention of photocarcinogenesis. Moreover, of tremendous significance is the maintenance of genomic stability in human melanocytes, which is afforded primarily by efficient DNA repair that is crucial for prevention of carcinogenesis.

An important finding is that the melanocortins α-MSH and ACTH, and ET-1 have survival effects on human melanocytes (10). These factors rescue cultured human melanocytes from apoptosis after UVR exposure or treatment with hydrogen peroxide. Increased survival of melanocytes in response to melanocortins and ET-1 is not dependent on increased melanin synthesis, as this effect was evident in tyrosinase-negative albino melanocytes that lack the ability to synthesize melanin. Other paracrine factors that act as survival factors for human melanocytes include NGF and MGF (11, 13, 97). The former is a keratinocyte-derived factor that reduces UVR-induced apoptosis by reversing the inhibitory effect of UVR on Bcl2 levels, and whose production is increased following exposure to UVR. MGF is the ligand for c-kit, and is essential for melanocyte survival and migration during embryonic development (98). MGF is also required for the survival of adult melanocytes, and its expression is up regulated by UVR (13, 99). These two survival factors, as well as the melanocortins and ET-1, activate inositol triphosphate kinase (IP3K) and its substrate Akt (10). Activated Akt signals for cell survival by inhibiting the mitochondrial apoptotic pathway, via phosphorylation of Bad, which inhibits its association with Bax (100).

That Akt is activated by melanocyte-specific survival factors suggests that it is critical for human melanocyte survival. In B-16 melanoma cells, MSH was shown to activate Mitf via phosphorylating and activating the upstream transcription factor CREB (101). As mentioned earlier, in human melanocytes, ET-1 phosphorylates CREB on Ser 133, and in the concomitant presence of MSH, CREB phosphorylation is further enhanced (82). Recently, we showed that MSH and ET-1 phosphorylate Mitf and inhibit the UVR-induced reduction in Bcl2 levels (10). The ability of Akt to activate CREB, one of its several downstream targets (102), and of CREB to phosphorylate and activate Mitf, links Akt activation to Mitf, the master regulator of melanocyte survival and function.

Importantly, the survival effects of melanocortins and ET-1 are accompanied by enhancement of repair of DNA photoproducts and reduced release of hydrogen peroxide following exposure to UVR (10). These effects are expected to reduce the burden of UVR-induced DNA damage, alleviate oxidative stress, and maintain genomic stability of melanocytes, thus reducing the risk for melanoma. Additionally, these findings ascribe a novel role for melanocortins and ET-1 as survival factors that enable melanocytes to overcome UVR-induced damage and inhibit mutagenesis. Whether or not other survival factors link survival mechanisms to DNA repair and antioxidant pathways remains to be determined. Given the sequence of events that are activated by melanocortins and endothelin-1, it can be concluded that repair of DNA and regulation of the redox state precede the increase in melanin content in human melanocytes and represent the first and early defense mechanism against apoptosis and genomic instability. Stimulation of melanogenesis by melanocortins and ET-1 occurs as a delayed response that protects the melanocyte, as well as the entire epidermis, against damage that might be caused by subsequent UVR exposures.

8. THE MC1R GENE AS A MELANOMA SUSCEPTIBILTY GENE

Epidemiological studies have linked some alleles of the *MC1R* gene with poor tanning ability and increased risk for melanoma. A study carried out in Great Britain found Asp84Glu substitution to be strongly associated with melanoma (22). A subsequent study from Australia, which included 460 melanoma patients and 399 control individuals, found that individuals carrying R151C, R160W, or D294H variant, known to be highly associated with red hair phenotype, had double the risk for melanoma, while having two of those variants increased the risk to 4 fold (21). Interestingly, this association with melanoma risk persisted among individuals with medium or olive/dark skin, suggesting that it is independent of skin color, and that the pigmentary phenotype is not always an accurate or reliable predictor of melanoma susceptibility. In this study, no independent association was found between melanoma risk and V60L or D84E variants. A third population study

from the Netherland found that expression of V60L, V92M, R142H, R151C, R160W, R163Q, and H260P in the homozygous or compound heterozygous state resulted in an odds ratio of 4 to develop melanoma, while expression of only one of those variants had an odds ratio of 2, further suggesting a gene-dosage effect (23). That V60L, V92M, and R163Q are not strongly associated with red hair or fair skin further confirms that melanoma risk is independent of pigmentary phenotype. A study that included 108 melanoma patients and 105 control subjects from France found that the frequency of functional *MC1R* variants was more than two fold higher in the melanoma than the control population, and that there was a gene dosage effect on melanoma risk (25). This study found three variants V60L, R151C, and R160W to be statistically more frequent in the melanoma group and to result in the highest risk for melanoma. A recent study conducted on a Mediterranean population concluded that expression of any *MC1R* variant, regardless whether or not it is associated with red hair, increased melanoma risk by 2 to 3 fold, compared to expression of the consensus sequence. This increased risk remained significant even after accounting for other known risk factors for melanoma, such as hair and skin color, tanning ability, number of nevi or presence of dysplastic nevi (26). An intriguing finding in this study is that *MC1R* variants in this population were statistically significantly associated with melanoma thickness, either due to delayed detection, or more aggressive tumors. Collectively, these studies unequivocally demonstrate the role of the *MC1R* in melanoma susceptibility. The strong association between *MC1R* variants and melanoma risk in individuals who do not have other important well-known risk factors, such as red hair and fair skin, high number of nevi and/or the presence of dysplastic nevi, suggests other functions for the MC1R, such as activation of DNA repair and antioxidant pathways, in addition to regulation of pigmentation.

We have been interested in elucidating how different *MC1R* variants affect the behavior of the melanocytes and their response to UVR. Using cultured human melanocytes that naturally express the *MC1R* gene, we found that the MC1R allelic variants R160W, R151C, D294H, R142H, and V60L are loss-of-function mutations in the gene (15, 94). Pharmacological studies on cells transfected with each of these variants corroborated our findings and revealed that these *MC1R* alleles do indeed disrupt the ability of the receptor to bind and/or to signal in the presence of ligand (27). Inability of human melanocytes that harbor these mutations to respond to melanocortins compromises their ability to activate nucleotide excision repair and reduce the level of hydrogen peroxide that is released following UVR irradiation. Production of hydrogen peroxide is expected to cause oxidative DNA damage, which together with unrepaired DNA photoproducts, is expected to increase the chance of mutations and carcinogenesis. We found that regulation of human melanocyte survival, DNA repair, and oxidative stress by the MC1R is independent of constitutive pigmentation, as human melanocytes with high total melanin and eumelanin contents that express loss-of-function MC1R have impaired DNA repair capacity and antioxidant defenses (10, 94). These findings are in agreement with epidemiological studies that concluded that pigmentary phenotype is not a reliable indicator of melanoma risk. Our results offer an explanation for the association of these *MC1R* alleles with increased risk for melanoma, and lend further evidence for the notion that activation of the MC1R by melanocortins is critical for melanoma prevention. Since ET-1 has similar effects as MSH on the response of melanocytes to UVR, we suggest that responsiveness to melanocortins and ET-1 is a factor that determines the susceptibility to melanoma. However, unlike the *MC1R* gene, there is no evidence so far for the association of loss-of-function mutations in the ET-B receptor gene with melanoma.

9. OTHER MELANOMA SUSCEPTIBILTY GENES

Beside the *MC1R* gene, the pigmentary gene *OCA2* or *P-gene* influences the risk for melanoma. OCA2 has been shown to be a determinant of eye color, and to influence the proliferation and differentiation of mouse melanoblasts, as well as melanosome biogenesis and eumelanin synthesis (103-104). The p-protein modulates the levels of intracellular glutathione and increases the sensitivity to arsenicals that cause oxidative stress (105). In a large study on Australian twin families, it was found that the effects of the *MC1R* genotype on skin color, freckling and nevus count were modified by eye color (106). Individuals with 2 strong red hair variants of the *MC1R* (which included D84E, R151C, R160W, and D294H) and brown eyes had twice the number of nevi as individuals with similar *MC1R* genotype and blue eyes. The association of *OCA2* and melanoma was confirmed by a study on melanoma patients and normal controls in France (107). The known functions of OCA2, mainly those related to regulation of eumelanin synthesis and increased sensitivity to oxidative stress, offer an explanation for how certain variants of the *P-gene* might affect melanoma susceptibility (figure 4).

An important melanoma susceptibility genetic locus is the *CDKN2A* (108-109). This locus codes for two tumor suppressors, $p16^{INK4a}$ (p16) and $p14^{ARF}$ (ARF) (110). The association of *CDKN2A* with melanoma susceptibility was based on the findings that germline mutations that disrupt the function or caused deletions of p16, and less commonly ARF, are expressed in melanoma kindreds (111-113). Somatic mutations in p16 were also found in sporadic melanomas, and were consistent with UVB-type signature mutations (74).The tumor suppressor p16 inhibits the CDk4-6-mediated phosphorylation of the retinoblastoma protein (pRb), thus maintaining its association with E2F (114). The binding of pRb to E2F inhibits the latter from translocating to the nucleus and suppresses its transcriptional activity (115). This function of p16 results in G1 arrest that is required for repair of DNA damage (116), and mutations in the p16 gene leads to uncontrolled cellular proliferation and impaired DNA repair. Mutations in the *CDK4* gene have also been found in some families with melanoma (117-118). That mutations in p16 and CDK4 are mutually exclusive indicates that they lie within the same signaling pathway. ARF (alternative reading frame), the second product of *CDKN2A* when the gene is

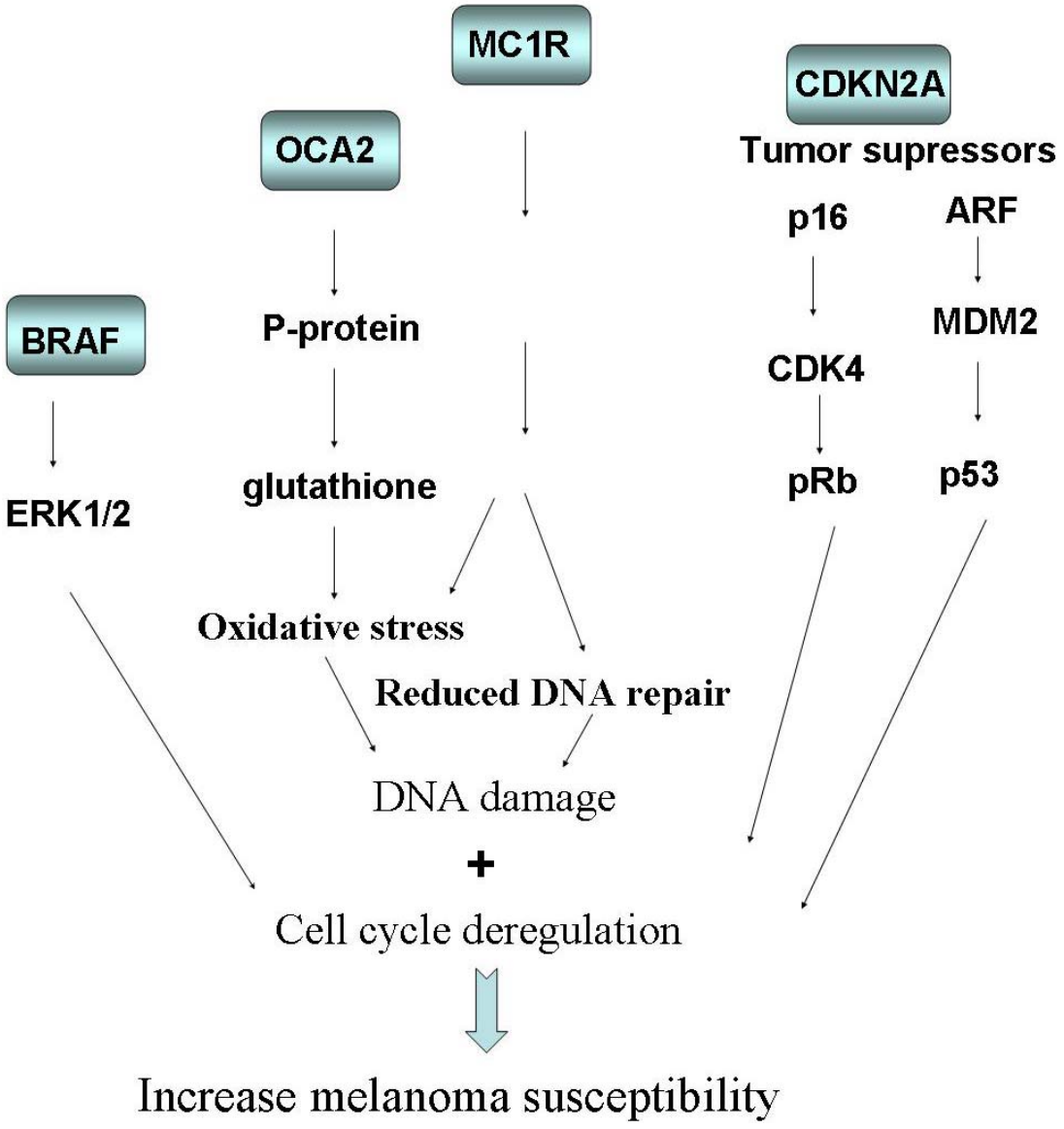

Figure 4. Important melanoma susceptibility genes and their functions in human melanocytes. The *CDKN2A* locus codes for the tumor suppressors p16 and ARF that regulate cell cycle progression and DNA repair via activating pRb, and the p53 pathway via inhibiting the activity of MDM2, respectively. Mutations that inactivate or delete p16, and less commonly ARF, are associated with increased melanoma susceptibility. The *MC1R* gene codes for the melanocortin 1 receptor, known to regulate eumelanin synthesis, and more recently, to enhance DNA repair and reduce oxidative stress. Certain allelic variants of this gene are strongly associated with melanoma independent of pigmentary phenotype. Expression of these *MC1R* alleles increases the penetrance of *CDKN2A* mutations, resulting in a higher risk and earlier onset of melanoma. The pigmentary gene, *OCA2*, codes for the p-protein, known to be particularly important in determining eye color. The p-protein regulates the level of glutathione in melanocytes, thus determines the extent of oxidative stress. Certain alleles of *OCA2* increase melanoma risk of individuals expressing melanoma-associated *MC1R* variants. The *BRAF* gene is important for regulating the ERK1/2 activity, and hence, proliferation. An activating mutation in this gene is common in melanoma tumors, and results in uncontrolled proliferation and maintenance of the transformed phenotype.

expressed using an alternative reading frame, regulates p53 function (119). ARF binds MDM2 and inhibits it from ubiquitinating and degrading p53 (119-120). Since p53 plays an important role as a sensor of DNA damage, cell cycle checkpoint and regulator of apoptosis, as well as DNA repair (116, 121-122), mutations that disrupt the function or expression of ARF are expected to lead to deregulation of the p53 pathway and its associated functions. Mutations in *CDKN2A*, but not in *RB* or *p53*, are prevalent in melanoma, where p16 and ARF seem to be

the "weakest link" that leads to pRb and p53 inactivation, respectively (123) (figure 4). Mutations in *CDKN2A* and *MC1R* gene are not mutually exclusive. Epidemiological studies have indicated that certain variants of the *MC1R* gene increase the penetrance of *CDKN2A* mutations, and further increase the risk for melanoma (24, 124). Individuals harboring mutations in both genes have been shown to have a higher incidence and earlier onset of melanoma tumors than individuals having mutations in either gene (figure 4).

Another gene that has been implicated in melanoma is *BRAF*. Constitutive activation of *BRAF* due to a single amino acid substitution, V599E, has been detected in
60 % of melanoma tumors tested (125). BRAF regulates the MAP kinases ERK1/2 that are necessary for melanocyte proliferation (81-82, 126). Constitutively active BRAF proved to be necessary for melanoma cell survival and transformation, as RNA silencing of $BRAF^{V599E}$ in melanoma cells results in their apoptosis, and loss of the transformation phenotype, as evidenced by their inability to form colonies in soft agar (127). Whether or not concomitant expression of $BRAF^{V599E}$, which deregulates proliferation, and a melanoma-associated *MC1R* variant, which reduces DNA repair capacity, contributes further to the risk for melanoma is not yet known (figure 4).

10. A NEW STRATEGY FOR MELANOMA PREVENTION

Melanoma, the most fatal form of skin cancer, arises from the malignant transformation of melanocytes. Unless detected early, melanoma tumors have a poor prognosis due to their high metastatic potential and resistance to conventional chemotherapeutic regimens. Since treatment of melanoma poses a challenge to clinicians, strong emphasis has been placed on its prevention and early detection. Prevention of melanoma involves defining tumor-specific genetic markers, which include susceptibility genes that predispose certain individuals to melanoma. Genetic markers should allow for the identification of individuals with a high risk for the disease. Routine follow up of these individuals is expected to lead to prevention and early detection of tumors. Melanoma prevention is an important public health goal, given the epidemic increase in the new cases that are diagnosed annually. In the U.S.A., the incidence of melanoma continues to increase at the rate of 4% annually, and its prevalence is increasing in individuals in the second and third decades of their lives; one in 75 persons is expected to develop melanoma at some point in his or her life (128).

We are developing a novel strategy for melanoma prevention based on the role of the MC1R gene as a melanoma susceptibility gene, and on the newly discovered functions of MSH as a survival factor for, and enhancer of DNA repair in, human melanocytes. We have tested newly designed potent synthetic analogs of MSH based on N-capping of the tetrapeptide His-D-Phe-Arg-Trp-NH_2, and showed that they can potentially be used for melanoma and non-melanoma skin cancer prevention (129-130). Previous structure-function studies revealed that the active site for MSH that is critical for its melanogenic effect is the tetrapeptide His-Phe-Arg-Trp, which constitutes the 6-9 amino acid sequence in the MSH tri-decapeptide (131-132). We have found that the tetrapeptide His-D-Phe-Arg-Trp-NH_2 is slightly less potent than MSH in stimulating tyrosinase activity of human melanocytes. N-capping of this tetrapeptide to yield 4-phenyl-butyryl-His-D-Phe-Arg-Trp-NH_2 or n-pentacedanoyl-His-D-Phe-Arg-Trp-NH_2 resulted in superpotent analogs with significantly lower EC_{50} values and more prolonged effect on tyrosinase activity than the native hormone MSH. These two analogs had a greater effect than MSH on the survival of UVR-irradiated human melanocytes, and mimicked the effect of MSH on repair of DNA photoproducts and reduction of hydrogen peroxide release. Both analogs are agonists of the MC1R, as their stimulatory effects on tyrosinase activity were markedly inhibited by an analog of ASP, and were not evident in human melanocytes expressing loss-of-function MC1R. Due to the small size and lipophilicity of these analogs, we expect them to be potentially useful as topical agents for prevention of melanoma. We expect these analogs to be of particular benefit to high-risk individuals that are heterozygous for one *MC1R* variant, or expressing mutations in melanoma susceptibility genes other than *MC1R*.

11. FUTURE CHALLENGES AND PERSPECTIVES

Recently, significant advances have been made in identifying melanoma susceptibility genes, some of which impact DNA repair pathways. Understanding the functions of these genes will undoubtedly unravel the mechanism(s) of melanocyte transformation to melanoma. Although pigmentary phenotype remains an important risk factor for melanoma, other factors need to be taken into account, mainly DNA repair efficiency, to insure accurate risk assessment. It is becoming more evident that pigmentary phenotype does not always accurately predict melanoma risk, and mutations in certain genes, such as the *MC1R*, in the absence of other risk factors predispose individuals to this cancer (21, 23, 26). These epidemiological results, together with our recent findings that the repair of CPD is compromised and release of hydrogen peroxide is accentuated in UVR-irradiated melanocytes that harbor loss-of-function MC1R (10), suggest the importance of repair of DNA photoproducts and antioxidant defenses in reducing UVR-induced genotoxicity and melanoma.

Further understanding of the response of human melanocytes to UVR is required, as important questions are still pending. For example, what are the chemical nature and properties of melanin produced by UVR-irradiated melanocytes, which accounts for the tanning effect of UVR? A recent study comparing the cutaneous responses *in vivo* of individuals from different ethnic backgrounds to UVR reported that despite increased expression of the melanogenic proteins tyrosinase, Tyrp-1 and –2, silver protein and Mitf, melanin content was only slightly increased following UVR exposure (133). It is conceivable that the tanning response to UVR is the outcome of the

"switch" towards preferential eumelanin synthesis in individuals with different pigmentary phenotypes, a possibility that requires investigation. A second question that needs to be addressed is whether or not paracrine factors and/or their respective receptors are differentially expressed in individuals with different pigmentary phenotypes, constitutively or in response to UVR. The importance of UVR-induced paracrine factors and their receptors, particularly melanocortins and the MC1R, in the response of melanocytes to UVR and their susceptibility to malignant tranformation is becoming more and more evident. Identification of genetic and epigenetic alterations that affect the responses of melanocytes from different skin types to these factors, and hence to UVR, is critical for understanding the differential susceptibility to melanoma, and for the design of effective strategies for melanoma prevention.

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