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Different types of DNA damage play different roles in the etiology of sunlight-induced melanoma

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The relationship between DNA damage, mutations and the initiation and progression of sunlight-induced melanoma has not yet been resolved. Recently, it has become apparent that the etiology of sunlight-induced melanoma is fundamentally different from sunlight-induced carcinoma, although both appear to be dependent on specific photoproducts produced in DNA by ultraviolet radiation (UVR). How DNA photoproducts are involved in the unique etiology of cutaneous malignant melanoma (CMM) compared to other types of skin cancer is central to understanding the nature of this disease and its prevention. Based on our results and published data, we propose a model for melanoma distinct from carcinoma, in which different types of DNA damage contribute to different stages of tumorigenesis.

Epidemiological observations of human skin cancer as well as data from experimental animal models offers considerable insight into skin cancer etiology and the DNA damage involved. Because human CMM develops on parts of the body exposed to chronic (head and neck) as well as sporadic sunlight (trunk), divergent etiologies have been proposed (Walker, 2008). Murine and piscine melanoma models expose neonatal animals to a small number of erythral UVB doses after which they receive no additional UVR. This is analogous to the “intermittent” or “sporadic” early childhood exposures often associated with truncal melanomas and differs from the “chronic” exposure history more closely associated with carcinoma and head and neck CMM. Additionally, in response to transgenic manipulation or interspecific hybridization, animal models acquire genetic factors that predispose them to CMM that mimic more the human UV-dependent truncal melanomas rather than the head and neck melanomas that are presumably associated with chronic sunlight exposure. The model we present here addresses truncal CMM that develop in response to early life (intermittent) exposure to UVR.

Using *Xiphophorus* first generation backcross hybrids we recently demonstrated that intermittent early life exposures to UVB but not UVA induce CMM in these fishes (Mitchell *et al.* 2010) (Fig. 1A). This result corroborates the wavelength-dependence of melanoma found in mammalian models; that is, UVA does not induce melanomas after neonatal exposure in either the South American opossum (*Monodelphis domestica*) (Robinson *et al.*, 1998) or hepatocyte growth factor/scatter factor (HGF/SF) transgenic mice (De Fabo *et al.*, 2004), both of which are susceptible to UVB-induced melanomagenesis. The most abundant damage induced by UVB irradiation results from the direct absorption of photons by DNA and includes the formation of cyclobutane pyrimidine dimers (CPD) and pyrimidine(6-4) pyrimidone dimers [(6-4)PD]. Within *Xiphophorus*, the amounts of thymine-cytosine CPDs and (6-4)PDs present in pigmented skin cells immediately after UVB irradiation are about the same (Fig. 1). Furthermore, the rate of removal of these lesions by photoreactivation is comparable and proportional to the reduction in melanoma frequency after the same light

treatment. If UVB exposed animals are allowed access to light wavelengths required for photoenzymatic repair, the incidence of CMM is dramatically reduced (Ley et al., 1989; Setlow et al., 1993; Setlow et al., 1989). Thus, in several animal models, the brief appearance of these photoproducts during early development appears to be necessary and sufficient to induce CMM.

Recently, it was demonstrated that topical treatment with a CPD-specific endonuclease immediately after UVB irradiation does not reduce the frequency of CMM in a *CDK4/NRAS* mouse melanoma model (Hacker et al., 2009) although similar treatments inhibit carcinoma formation in mouse models (Bito et al., 1995; Yarosh et al., 1992). These studies suggest that the (6-4)PD and CPD may affect the etiology of melanoma and carcinoma in different ways. This hypothesis is supported by the fact that, although UVA is a complete carcinogen in mouse carcinoma (de Laat et al., 1997; Kelfkens et al., 1991), it does not induce melanomas in animal models (De Fabo et al., 2004; Mitchell et al.; Robinson et al., 2000). The presence of C→T signature mutations in DNA from chronically-UVA induced carcinomas is consistent with the observation that UVA induces CPDs at a greater frequency than oxidative damage (Mouret et al., 2006). The fact that UVA does not induce (6-4)PDs (Mouret et al.), has a minimal effect on gene expression in melanocytes (van Schanke et al., 2005) and does not induce melanoma may not be coincidental.

Structural differences between the CPD and (6-4)PD determine their biochemistry and ultimately how these lesions are involved in sunlight-induced skin cancer (Mitchell and Nairn, 1989; Reardon and Sancar, 2005). The (6-4)PD produces a 43° bend in the DNA helix with the 3' base lying at 90° relative to the 5' base. In contrast, the pyrimidines comprising the CPD are stacked quasi-parallel to one another and produce only a 7° bend in the DNA helix. Hence, the (6-4)PD is considerably more distortive than the CPD and considerably more effective at blocking the progression of replication and transcription polymerases. The relative mutagenic potential of CPDs and (6-4)PDs was examined in NER-deficient human cells using CPD photolyase (Asahina et al., 1999; Marionnet et al., 1998) and elegantly addressed in hairless mice using CPD and (6-4)PD photolyases (Garinis et al., 2006; Jans et al., 2005). In contrast to the (6-4)PD, which had no effect on mutations, specific removal of the CPD greatly reduced the frequency of signature mutations, demonstrating that the CPD is the predominant mutagenic lesion.

Squamous and basal cell carcinomas are characterized by a significant occurrence of C→T and CC→TT transition mutations in RAS oncogenes, as well as in p53 and PTCH tumor suppressor genes (Sarasin, 1999). Such signature mutations can be linked to sites of T-C pyrimidine dimer formation and are the “smoking gun” for sunlight-induced carcinoma (Ziegler et al., 1993). Indeed, the vast majority of UVB-induced mutations are associated with CPDs (You et al., 2001). The paucity of these CPD-derived mutations in melanoma, particularly truncal melanomas, is problematic and suggests that mechanisms other than the mutation-driven inactivation of tumor suppressor genes may be more relevant to melanoma initiation. Indeed, melanomas induced after neonatal UVB irradiation in *Xiphophorus* backcross hybrids lack UV signature mutations in *Tp53* (Kazianis et al., 1998).

Two recent studies that associate UVB signature mutations with melanoma would appear to contradict our hypothesis. A high frequency of UV signature mutations has been seen in a catalogue of ~32,000 somatic mutations from a melanoma cell line (COLO-829) (Plesance et al., 2009). Unfortunately, no skin primary melanoma was identified and the high number of C→T and CC→TT transitions may reflect the location of the primary tumor in a sun-exposed site receiving chronic UVR. There is also a recent study showing significant frequencies of C→T mutations in the PTEN gene in melanomas from various body sites (including the trunk) from a *xeroderma pigmentosum* patient population (Wang et al., 2010).

Results from repair-deficient patients are difficult to translate to the general population since the pre-mutagenic lesions (both CPDs and (6-4)PDs) are not repaired and can persist in surviving skin cells indefinitely. Although photoproducts originally induced by solar UVR are diluted by cell division and tumor growth, they are not repaired and would resemble persistent damage induced by chronic low-dose exposure in a repair-proficient individual. Since the nucleotide excision repair capacity of melanoma cells is at least as proficient as melanocytes (Gaddameedhi *et al.*, 2010; Hatton *et al.*, 1995), progression would neither affect repair capacity nor the number of potentially mutagenic lesions. Hence, in both of these studies, accumulation of signature mutations may not be associated with initiation but may result from the continuous exposure of incipient (initiated) melanomas to sunlight and the dysregulation of cell proliferation associated with progression (see below).

If direct photoreactivable damage is required for melanoma and CPDs are not involved, then, it is worthwhile to examine the unique properties of the (6-4)PD. In contrast to the CPD, which forms uniformly in chromatin, the (6-4)PD is induced at a significantly greater frequency (~6-fold) in linker compared to nucleosome DNA (Mitchell *et al.*, 1990), suggesting that more open chromatin configurations, such as transcribing genes and regulatory elements (promoters), are considerably more vulnerable to (6-4)PD induction than condensed heterochromatin. Both CPDs and (6-4)PDs can inhibit DNA-protein interactions, as well as *in vitro* transcript production and transactivation of reporter genes (Ghosh *et al.*, 2003). DNA damage in a promoter element (*e.g.*, Sp1, AP-1, NF κ B) can inhibit or enhance binding transcription factor (TF) resulting in either an increase or decrease in gene expression (Mitchell and Ghosh, 2007). In addition, UV photoproduct hotspots *in vivo* are associated with TF binding to promoters. Specifically, (6-4)PD formation is significantly enhanced in the CCAAT box upstream from the *PGK1*, *JUN* and *PCNA* genes (Pfeifer *et al.*, 1992), the TATA box domain of the active *SNR6* and *GAL10* genes (Aboussekhra and Thoma, 1999) and in the p50-bound NF κ B gene (Ghosh, 2001; Ghosh *et al.*, 2001).

CPDs and (6-4)PDs preferentially form at 5'T^{met}C and 5'T^{met}CG sequences (Tommasi *et al.*, 1997; Mitchell, 2000; You and Pfeifer, 2001). This is of particular interest since CpG islands are prominent in promoter regions and are intimately involved in methylation status and gene regulation. Preferential (6-4)PD induction at CpG islands could alter gene expression patterns by inhibiting DNA methyltransferases, increase aberrant hypermethylation and repress gene expression (Howell *et al.*, 2009). On the other hand, the helical distortion associated with the (6-4)PD can mimic a TF binding motif, thus either increasing gene expression if located within a promoter element or reducing gene expression through molecular hijacking (Mitchell and Ghosh, 2007). The high affinity binding of high mobility group 1 (HMG1) protein to oligonucleotides containing (6-4)PDs (Pasheva *et al.*, 1998) and inhibition of nucleosome assembly by (6-4)PDs (Matsumoto *et al.*, 1995) may also reflect potential effects of these photoproducts on DNA (gene) accessibility. Ultimately, changes in gene expression can have a significant impact on signaling cascades and result in auto-regulation and permanent heritable changes in the pre-neoplastic cells involved in tumorigenesis (MacLeod, 1996).

Immediately after UV exposure, DNA replication is inhibited in a dose-dependent manner and recovers as a consequence of NER (reviewed by (Cleaver and Mitchell, 2003). The potency of the (6-4)PD as a structural block to replication and transcription, its ability to signal cell cycle arrest (Lo *et al.*, 2005) and the correlation between the recovery of replication and (6-4)PD excision kinetics (Griffiths *et al.*, 1990), suggests that the inhibition of DNA synthesis and activation of signal cascades associated with replication arrest is a major consequence of the (6-4)PD. Given the fact that stalled replication forks are the predominant DNA damage response signals, it stands to reason that the (6-4)PD may be

responsible for significant changes in gene expression within the first few hours post-irradiation. If early changes in gene expression are important in melanocytic transformation (i.e., initiation) and if such changes are signaled through stalled replication forks, then it is likely that the (6-4)PD may be the primary structural determinant for initiating UV-induced intermittent melanoma. Transient increases and decreases in the expression of a number of genes shortly after UVB has been observed in several different human and mouse systems (Greinert, 2009; Tyrrell, 1996). Replication forks blocked at sites of (6-4)PDs may have the capacity to initiate signal transduction cascades through p16/INK4a or a receptor tyrosine kinase/RAS pathway that can affect mitogenic signaling and ultimately transcriptional regulation by pRB (de Gruijl et al., 2005).

Our model does not preclude a role for reactive oxygen species (ROS) in intermittent or chronic melanoma development. The inability of melanocytes to repair DNA lesions associated with UVA-induced oxidative damage may contribute to progression of chronic melanomas (Wang et al.), particularly in male fish (Joose *et al.*, 2010). The proportion of oxidized purines produced by UVA or UVB predominates over the relatively few oxidized pyrimidines (Kielbassa et al., 1997; Pouget *et al.*, 2000). Hence, the T→A transversion in *BRAF*, a hallmark of truncal melanoma (Davies *et al.*, 2002) would be a fairly rare sunlight-induced mutation, although an innovative pathway to the T→A transversion in *BRAF* has been proposed based on error-prone replication of DNA damage proximal to the site of the V600 mutation (Thomas et al., 2006). In the absence of chronic UVR, truncal melanomas, initiated by the (6-4)PD, could acquire mutations through endogenous free radical mechanisms (Fig. 2). As initiated cells progress toward malignancy, the accumulated loss of growth controls and increased genetic instability would serve to increase endogenous free radicals and oxidative lesions in DNA that can further damage and dysregulate melanocyte proliferation. This self-perpetuating cycle could account for the high *BRAF*V600 mutations found in truncal melanomas.

Does this model for the initiation of truncal melanoma have any relevance to the etiology of melanomas found on chronically sun-exposed sites? It is very likely that the early life erythral events associated with intermittent melanoma are not restricted to the trunk but also occur on body sites associated with chronic sunlight exposure. Hence, one would expect a similar etiology for initiation of both intermittent and chronic melanomas with extended exposure of initiated melanocytes to sunlight and DNA photoproducts serving to accelerate progression and increase malignancy in sun-exposed areas. Indeed, C→T signature mutations are found in considerably higher frequencies in melanomas from sun-exposed surfaces (Walker, 2008) and, although not involved in initiation, could affect tumor progression. Importantly, animal models do not support a role for chronic UV exposure in the induction of melanoma; chronic exposure to UVB does not induce melanoma in the HGF/SF transgenic mouse (Noonan et al., 2000) and, whereas a single high dose of UVB can stimulate melanocyte proliferation in mouse skin, a fractionated exposure has no effect (van Schanke et al., 2005). Therefore, unlike carcinoma, protracted exposure to UVR is not effective in melanoma induction in these mammalian models. These experiments need verification in other melanoma models (*e.g.*, fish) but suggest that continued exposure of initiated melanocytes to UVR may actually mitigate progression of these cells to melanoma.

In summary, we believe that the different photoproducts induced in DNA by solar UVR have different consequences, depending on the site and mode of exposure. It is fairly well accepted that there are significant differences in the etiologies of carcinoma and melanoma. Carcinoma is considered a mutation-driven skin cancer associated with extensive chronic sunlight exposure and exhibits expected UV signature mutations in known tumor suppressor genes (*e.g.*, p53). Melanoma, on the other hand, appears to have multiple etiologies depending on where the tumor occurs, how much sunlight exposure it receives and genetic

predisposition. In response, divergent pathways have been suggested for chronically-exposed and intermittently-exposed melanomas. However, as animal models demonstrate, the role of chronic exposure in the induction of melanoma is problematic.

We have addressed the relationship between DNA damage and melanoma and suggest that the (6-4)PD may be a major player in its initiation. Furthermore, we suggest that (6-4)PD-mediated initiation may not be mutation-driven but rather has the potential to elicit epigenetic responses that result in shifts from transient to permanent changes in melanocyte gene expression patterns (Howell *et al.*, 2009). It should be kept in mind that the biological effects of the CPD and (6-4)PD are qualitatively the same; that is, the CPD can block DNA synthesis and the (6-4)PD can be mutagenic, particularly in rapidly proliferating systems (Wood, 1985; Franklin and Haseltine, 1986; Lawrence *et al.*, 1993). What distinguish the molecular consequences of these lesions, whether lethal or mutagenic, are their profound differences in structure. Hence, in Fig. 2 we show the preferred pathways for these two photoreactivable lesions in higher vertebrates (e.g., fish and mammals), aware of the fact that their effects may overlap, although the mouse photoreactivation experiments (Garinis *et al.*, 2006) would suggest otherwise (see above). We do not exclude the role of other types of DNA damage (*i.e.*, CPDs and oxidative lesions) or mutagenesis in melanoma progression, but consider that many of the mutations may be a consequence rather than a cause of melanomagenesis.

The importance of the (6-4)PD in melanoma needs to be directly examined. Animal models that selectively express CPD and (6-4)PD photolyases may provide a fruitful platform, perhaps the only platform, for testing these ideas. Incorporating damage-specific photolyase genes into a mouse melanoma model or creating a CPD and/or (6-4)PD photolyase knockdown in a *Danio* or *Medaka* fish melanoma model are warranted. Affirmation of the (6-4)PD as a key initiator would significantly alter our perception of sunlight-induced melanoma, clarify some of the major discrepancies in the relationship between sunlight and melanoma and offer new strategies for risk assessment and prevention.

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SIGNIFICANCE

Sunlight-induced melanoma is widely regarded as a highly heterogeneous cancer with diverse etiologies. Here we show that a specific class of DNA damage associated with a specific wavelength region of solar ultraviolet radiation is necessary for melanoma initiation. We propose a pathway that precludes a role for mutagenesis and suggests that oxidative damage produced endogenously or enhanced by chronic sunlight can not initiate melanoma but may factor into the progression and severity of the disease. Based on our knowledge of ultraviolet photochemistry and photobiology, we propose a unique mechanism for melanoma initiation and progression, with a particular emphasis on the pyrimidine-pyrimidone (6-4) photoproduct as a causative agent.

Fig. 1.

Correlation between direct damage in DNA and melanomagenesis in a backcross hybrid fish model. In Panel A the effect of photoenzymatic repair on melanoma is shown. Black bar shows UVB-induced melanoma, gray bar shows melanoma frequency in UVA-treated fish and open bars show effects of photoreactivation and background melanoma frequencies. Statistical analyses shows significant differences between UVB-induced melanoma and UVB+PRL, -UVR and UVA; there is no significant difference between UVB+PRL, -UVR and UVA. In Panel B the induction of T<=>C CPDs and T-C (6-4)PDs is shown in pigmented skin from *Sp-couchianus* F₁ hybrids after a single UVB treatment (black bars) and in response to photoreactivating light (open bars).

Fig. 2.

Hypothetical model for the differential effects of cyclobutane dimers and (6-4) photoproducts on the etiology and progression of malignant melanoma and carcinoma. Shown are separate pathways for the etiologies of carcinoma (left) and melanoma (right) and their relationships to the two photoreactivable lesions induced by solar UVA and UVB in the skin. Chronic prolonged exposures associated with carcinoma are distinguished from acute, intermittent early-life exposures associated with a (6-4)PD-dominated pathway. Multiple pathways are shown leading to changes (▲) in gene expression with the (6-4)PD showing a more diverse influence. Reactive oxygen species (ROS) are shown affecting progression for both carcinoma and melanoma; ROS may also be involved in metastasis.