Ultraviolet A does not induce melanomas in a *Xiphophorus* hybrid fish model

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We examined the wavelength dependence of ultraviolet (UV) radiation (UVR)-induced melanoma in a *Xiphophorus* backcross hybrid model previously reported to be susceptible to melanoma induction by ultraviolet A (UVA) and visible light. Whereas ultraviolet B (UVB) irradiation of neonates yielded high frequencies of melanomas in pigmented fish, UVA irradiation resulted in melanoma frequencies that were not significantly different from unirradiated fish. Spontaneous and UV-induced melanoma frequencies correlated with the degree of pigmentation as expected from previous studies, and the histopathology phenotypes of the melanomas were not found in significantly different proportions in UV-treated and -untreated tumor-bearing fish. Our results support the conclusion that a brief early-life exposure to UVB radiation causes melanoma formation in this animal model. These data are consistent with an essential role for direct DNA damage, including cyclobutane dimers and (6-4) photoproducts, in the etiology of melanoma.

ultraviolet B | DNA damage | cyclobutane dimer | reactive oxygen species | melanin

In the late 1980s Setlow and coworkers used genetic hybrids from interspecific crosses involving several species of the fish genus *Xiphophorus* to investigate the effects of UVR on the induction of cutaneous malignant melanoma (CMM) (1). These pioneering studies demonstrated that ultraviolet B (UVB) irradiation of backcross hybrids generated from a specific genetic crossing scheme induced melanomas at significant frequencies above spontaneous levels. These results were later confirmed, and the genetic basis of UVB-induced melanoma susceptibility in this cross was recognized to be the same as in the well-studied spontaneous *Xiphophorus* hybrid melanoma model (2). In 1993, Setlow used a different *Xiphophorus* interspecies cross (designated as Sp-couchianus; Fig. 1) to study the wavelength dependence of melanoma induction and reported that wavelengths in the ultraviolet A (UVA) and visible ranges were effective in inducing melanomas in first-generation backcross (BC₁) hybrids generated from this particular cross (3). An action spectrum for melanoma induction was proposed with maxima in the UVB (302/313 nm) and UVA (365 nm) ranges. Because UVA fluence is quantitatively much greater than UVB in sunlight incident to the earth's surface (~10-fold), Setlow suggested that, on the basis of this action spectrum, UVA was more effective than UVB in causing melanomas in the human population (2, 4). This report had significant public health consequences; it suggested that the use of commercially available sunscreens that effectively blocked UVB but not UVA encouraged more lengthy recreational sunlight exposure and thereby increased the exposure to UVA and its associated risks. Over the past 20 years, these data have become central to the debate on the role of UVA in melanoma and the risks associated with recreational and artificial exposures to UVA wavelengths.

Debate over the action spectrum for melanoma has only intensified because subsequent research using a variety of animal models has not corroborated these results. Studies in mammalian models, including the South American opossum (*Monodelphis*

domestica) and several genetically modified mouse models, demonstrate that UVA does not induce melanomas (5–7). In contrast, evidence from all of these models, including *Xiphophorus*, supports a role for UVB in the etiology of melanoma. Unlike placental mammals, fish are very efficient at removing UVB-induced direct damage in DNA using photoenzymatic repair (PER) (8, 9) and this mechanism greatly decreases melanoma formation in the *Xiphophorus* melanoma model (1, 3). 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 (6-4) pyrimidine dimers [(6-4)PD] (10, 11). Collectively, the results from the vast majority of animal studies strongly suggest that one (or both) of these lesions is required for melanoma formation.

Xiphophorus offers some significant advantages over other animal models for investigating melanoma. The classical, spontaneous *Xiphophorus* hybrid melanoma model has been studied for over 80 years and offers powerful genetic and biochemical approaches for revealing determinants of melanomagenesis (12–14). The *Xiphophorus* melanoma receptor kinase gene (*XMRK*) behaves as a dominant oncogene in this fish model and is a mutated derivative of the fish ortholog for the human epidermal growth factor receptor (EGFR/Erbb-1). Consistent with the activity of mammalian EGFR in melanocytes (15–17), activation of the *XMRK* oncoprotein leads to numerous downstream signaling cascades including, but not limited to, the RAS/RAF/MAPK and PI3-K/AKT signaling pathways (12). In addition to these signaling cascades, the transformed phenotype in *Xiphophorus* also involves participation of transcription factors (e.g., STAT5) and glycoproteins (e.g., osteopontin, OPN) that are intimately involved in cellular proliferation and antiapoptotic responses that characterize numerous human cancers. Hence, *Xiphophorus* hybrid models offer ideal experimental platforms to further elucidate the biochemistry underlying melanomagenesis within the context of a controlled genetic background.

A recent publication (18) exploited the same *Xiphophorus* Sp-couchianus hybrid melanoma model used by Setlow for UVA induction studies (3) to investigate photosensitization of melanin as a possible mechanism for melanoma formation. In this report, electron paramagnetic resonance was used to describe the UVR wavelength dependence of reactive melanin radical formation in pigmented fish skin. The action spectrum for melanin-sensitized generation of reactive radicals derived from these experiments positively correlated with the action spectrum for melanoma formation reported by Setlow (3). These results are consistent with a role for UVB and UVA in generating melanin-derived reactive

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Fig. 1. The *Sp-couchianus* backcross hybrid breeding scheme. F₁ hybrids are produced by mating a macromelanophore pigmented “spotted side” (*Sp/Sp*) *X. maculatus* female from strain Jp 163 B (Upper Left) to a *X. couchianus* (Xc) male not carrying the *Sp* allele (*+/+*) (Upper Right), therefore not exhibiting any macromelanophore pigmentation. F₁ hybrids are backcrossed to either male or female *X. couchianus* individuals and produce BC₁ progeny of which ~50% exhibit the *Sp* (spotted side) (*Sp/+*) and 50% the wild-type (*+/+*) pigment phenotypes.

oxygen species (ROS) in melanoma causation. However, the observation that virtually all other animal models developed to study melanoma are refractory to UVA-induced melanoma calls into question the role of melanin-derived ROS in melanoma induction.

With these considerations in mind, we used the *Xiphophorus Sp-couchianus* model in experiments designed to replicate the results of Setlow’s seminal UVA melanoma study (3), with specific attention to the requirement for sufficient sample sizes to allow statistical inferences. Our primary goal was to resolve the ongoing debate regarding the action spectrum for melanoma induction. Specifically, we wanted to address the following questions: (i) Does UVA induce melanomas in this model? (ii) Is there a correlation between the degree of genetically defined pigmentation and melanoma susceptibility? (iii) Are there any differences in the histopathologies of spontaneous and UVR-induced melanomas? By answering these questions we hope to increase our understanding of melanoma causation and the roles played by UVR and DNA damage in melanoma initiation.

Results

UVB but Not UVA Induced Melanomas in the *Sp-couchianus* Backcross Hybrid Model. On the basis of the initial UVR melanoma induction results using the *Sp-couchianus* model (3), we performed an approximate power calculation for a two-sample comparison assuming a background value of 15–20% and a 2- to 3-fold increase in melanoma after treatment. From this calculation we determined that 150–200 fish would be required to achieve statistical significance ($P < 0.005$). The sample sizes of each treatment group at the end of the experiment were 216 control (–UV) fish, 282 UVA irradiated fish, and 194 UVB irradiated fish. Because tumor latency in this particular hybrid cross can be prolonged, for completeness our study was designed to allow for tumor formation and progression through maturity, and fish were killed at 12–14 months of age. However, exophytic lesions were scored throughout the course of the experiment and typically occurred in fish >6 months of age with the majority of tumors arising between 8–12 months. The mortality rate during the experiment not due to melanoma formation was consistent across the three treatment groups (–UV = 10%, UVA = 6%, and UVB = 11%).

We found the incidence of melanomas to be significantly greater in the UVB-irradiated fish compared with the nonirradiated control fish (UVB: 86 tumor-bearing fish out of 194 individuals,

melanoma frequency = 44.3%; control: 40 tumor bearing fish out of 216 individuals, melanoma frequency = 18.5%; $\chi^2 = 31.99$, $P < 0.0001$). However, there was no difference in melanoma incidence between the UVA-irradiated fish and that of the control fish (UVA: 35 tumor-bearing fish out of 282 individuals, melanoma frequency = 12.4%; control: 40 tumor-bearing fish out of 216 individuals, melanoma frequency = 18.5%; $\chi^2 = 3.57$, $P > 0.05$). Therefore, our results contradict the report of Setlow and colleagues (3) and indicate that UVB but not UVA significantly induces melanomas in this *Xiphophorus* melanoma model (Fig. 2). We used doses of 6.4 and 80 kJ/m² UVB and UVA, respectively, for each of the five UV treatments; Setlow and colleagues used a dose range of 0.35–3.0 and 0.5–11.75 kJ/m² 313 (UVB) and 365 (UVA), respectively, for their treatments. Because of differences in emission spectra and dosimetry, these doses are not directly comparable. However, on the basis of our toxicity and DNA damage determinations, our incident doses are equal to or exceed those of Setlow et al. (3).

Exposure of Neonates to UVA or UVB Did Not Affect the Degree of Adult Pigmentation. The degree of pigmentation was visually estimated in each fish by two observers (L.P. and D.T.) as (i) light with 50–70% coverage, (ii) intermediate with 70–90% coverage, and (iii) heavy with >90% coverage. The distribution of pigmentation in the different experimental groups was determined at 4 months and at the time of sacrifice at 12–14 months (Table 1). From Table 1, it is clear that the amount of pigmentation an individual possessed increased with their age in all treatment groups. However, it should be noted that there did not appear to be any differences in the distribution of pigmentation among the treatment groups at the time of sacrifice. Hence, episodic neonatal exposure to either UVA or UVB did not influence the degree of pigmentation in the adult animals.

Degree of Adult Pigmentation Was Positively Correlated with the Frequency of Spontaneous and UV-Induced Melanomas. Melanoma frequencies were positively correlated with pigmentation in all of the treatment groups (Table 2). Approximately 90% of the spontaneous, UVA and UVB tumors occurred in fish with intermediate and heavily pigmented phenotypes. These data indicate that melanoma susceptibility in this animal model is dependent on the level of pigmentation. It should be noted that animals with more melanin pigmentation would also concomitantly increase the number the XMRK oncoproteins because melanocytes are their substrate. It

Fig. 2. Melanoma frequencies in unirradiated and UV-irradiated BC₁ pigmented fish. Melanoma frequencies (%) are shown for the –UV control, UVA, and UVB fish. A significant difference (**) was found between the UVB and both the –UV and UVA treatment groups ($P < 0.0001$). No difference was seen between the –UV and UVA treatment groups ($P > 0.05$).

Fig. 3. Spindle cell melanomas (SCM) stages I–IV. Stage I tumor shows lateral growth located entirely in the dermis without infiltration into the muscle bundles (A). Cross-section of a stage II tumor showing lateral growth with a small proportion of tumor cells infiltrating the underlying tissue (B). Stage III tumor showing more extensive invasion of the musculature (compared with stage II) (C) and stage IV exophytic melanoma shows extensive invasion into the muscles bundles (D). (Magnification: $\times 100$.)

from both sides in a UV-transparent chamber. Using this facile technique we could treat a large number of fish and accumulate animal numbers that would give us statistical confidence in our results. Second, we allowed our fish to develop tumors for 12–14 months as opposed to 4 months postirradiation. We believe that most of the potential spontaneous (background) and UV-induced melanomas would be fully expressed in older animals, thus avoiding any effects of latency on the melanoma frequency. Third, nearly all of the tumors scored were examined by histopathology and clearly shown to be melanomas of various histological types (Table 1 and Fig. 3).

In view of the disparity between the present results and those of Setlow and colleagues (3), it is informative to reconsider their results (as set out in their table 1 and figure 2 in the 1993 paper). We reviewed their action spectrum data using careful estimates graphically derived from their published dose–response curves (Table S1 and Fig. S1). In their study, two to six groups (depending on the number of available fish) of ~ 5 fish each were irradiated in a 1-cm quartz cuvette with vortex mixing. A background level of 24% was derived from 124 untreated fish and used for the UVB (302 and 313 nm) and UVA (365 nm) portion of the action spectrum; a different background level was used for 405- and 436-nm data (see below). On the basis of these results, and before our study, we performed an approximate power computation for a two-sample comparison using a melanoma incidence of 45% for the treated fish and 24% for the untreated fish. We determined that at least 150 fish were required for statistical reliability. For each of the two UVB (302 and 313 nm) and single UVA (365 nm) experiments the total number of fish was 123, 124, and 85, respectively. However, unlike the control experiment, the treated fish were subgrouped into multiple doses for the UVB, UVA, and blue light dose–response curves. Assuming that comparable numbers of animals were used at each dose level, the 302- and 313-nm UVB treatments would have consisted of ~ 31 fish per dose, the 365-nm UVA treatment of ~ 14 fish per dose, and the 405- and 436-nm treatments of ~ 15 and ~ 10 fish per dose, respectively. These sample sizes are considerably lower than what is

required for statistical inference to compare each dose to the control group.

We replotted close estimates of the values from the published 313-nm and 365-nm plots (figure 2 B and C in ref. 3) on a semilog plot (i.e., log fraction of fish with tumors vs. dose) (Fig. S1). Setlow suggested that the sensitivity of the pigmented *Xiphophorus* BC₁ fish to melanoma was consistent with single-hit kinetics and based his action spectrum on the k -values (slopes) derived from dose–response curves at each wavelength. Linearity on a semilog plot is characteristic of a single-hit response. Unfortunately, the dose–response curves shown in figure 2 of the 1993 paper (3) were not presented on semilog plots nor were any correlation coefficients associated with these lines given. From our reanalysis of the 313 and 365 dose–response curves (Figs. S1 and S2 and Table S1) we did not observe linearity on a semilog plot. Whereas the UVB dose–response appeared positive, the UVA response appeared to maintain a slope of 0 for doses > 0.5 kJ/m². The error bars for the melanoma frequency at 365 nm for the lowest dose extended below background and showed considerable overlap with the higher doses. It is possible that the melanoma response saturated at < 1 kJ/m², suggesting that UVA-induced melanoma is extremely sensitive to low doses. Our 353-nm dose was on the shoulder of the survival curve, hence was as high as we could use without killing substantial numbers of animals (i.e., doubling this dose resulted in considerable mortality). Hence, we believe that our UVA dose is at or greater than the 365-nm doses used by Setlow and colleagues (3). However, we saw no UVA-induced melanoma formation above background at this dose. Indeed, melanoma formation at low UVA doses is particularly disconcerting because melanoma formation is associated with high episodic sunlight exposures (i.e., sunburn) of young individuals.

Similar problems are evident for the blue light portion of the Setlow action spectrum (i.e., 405 and 436 nm). First, a small number of experimental animals were used; that is, 15 fish/dose for 405 (total = 61 fish) and 10 fish/dose for 436 nm (total = 21 fish). All of the melanoma frequencies at these wavelengths appear very similar to the original 24% background used for the UVB and

UV Exposure Protocol. Subsequent to treatment, fish from different broods born within a 2-week period were combined into large (55 gal) community tanks. Pigmented fish were easily identified at sexual maturity (~4 months of age) and segregated from nonpigmented fish. At this time, all pigmented fish were scored on the basis of their degree of melanin pigmentation (i.e., initial pigmentation, Table 1). The degree of pigmentation was estimated in each fish in the tumor study as heavy with >90% coverage, intermediate with 70–90% coverage, and light with 50–70% coverage. Interestingly, we observed relatively few tumors during the segregation of pigmented fish, which coincides with early adulthood in these fish and is within the time frame that Setlow and colleagues (2) scored their treatment groups for melanomas. For the remainder of the experiment, these pigmented fish, which have the *Xmrk* oncogene, were housed as small groups (four to six fish) in 5.5-gal tanks to facilitate observing tumors. All exophytic tumors were noted at the time of detection and a final count of all tumor-bearing fish was conducted at the end of the experiment (i.e., 12–14 months of age). Upon conclusion, fish were killed using a lethal dose of anesthesia (MS-222) and were preserved in 10% neutral buffered formalin. All tumor-bearing fish and heavily pigmented fish were sent to pathology for internal analysis and tumor identification once preserved.

Histology. Tumor-bearing fish were killed and the lesions and surrounding tissues removed by dissection. Depending on the size, the excised tumors were

divided in several pieces. NBF-fixed pieces of the tumors were embedded in paraffin wax and sectioned by conventional histology techniques. The slides were stained with hematoxylin and eosin. For each sample, slides were prepared from several different areas, so that we had a representative picture of the morphology of the entire tumor. Other pieces of these tumors were reserved for genetic characterization, which we hope to correlate with our histological observations in future studies. The histopathologies of *Xiphophorus* melanomas have been well categorized in a prior study (19) and, therefore, we have provided only a brief description of type and stage classifications. Tumor types in the *Xiphophorus* model include: SCM, characterized by the presence of fusiform spindle cells with elongated nuclei, confined to the dermis or invading the muscle bundles; MMM are characterized by the presence of many different cell types, including fusiform spindle, round, epithelioid cells as well as melanophores and macromelanophores cells; ECM containing cells that resemble epithelial cells; and MM, which are characterized by the presence of cells with dendritic morphology. The stages of the tumors were determined in all of the different melanoma phenotypes, taking into consideration the size and the invasiveness into the muscle bundles (*Results*).

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