Melanin Reduces Ultraviolet-Induced DNA Damage Formation and Killing Rate in Cultured Human Melanoma Cells

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Epidermal melanin pigment is believed to prevent development of ultraviolet (UV)-induced skin cancer by shielding cell nuclei and reducing DNA damage formation. It has not been experimentally proved, however, whether melanin reduces UV-induced DNA damage, because published experiments have been inconclusive. The present study was carried out to determine whether intracellular melanin protected cultured cells against UV-induced DNA damage and killing. Three human melanoma cell lines containing different amounts of melanin were used. Absorption spectrum, subcellular localization of melanin, and melanin concentration were examined in the three cell lines. Two types of DNA damage, cyclobutane pyrimidine dimers and (6-4)photoproducts, were detected by an enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies specific for these photolesions. We found that melanin reduced the induction rates of both types of DNA damage in pigmented cells irradiated with low doses of UV in a melanin concentration-dependent manner. Almost no differences in repair capacity for the two types of photolesions were observed among the three melanoma cell lines. We also found that the more highly melanotic melanoma cell lines were more UV resistant than the less melanotic melanoma cell lines. These results suggest that intracellular melanin plays an important role in preventing UV-induced cell killing by reducing the formation of two types of DNA damage. Key words: cyclobutane pyrimidine dimer/(6-4)photoproduct/monoclonal antibody/photoprotection. *J Invest Dermatol 101:685-689, 1993*

Epidemiologic studies have indicated that sunlight is one of the most important causes of skin cancer [1-4]. Ultraviolet (UV) irradiation induces DNA damage in epidermal cells [5], which is potentially carcinogenic [6]. Highly pigmented races are known to be much less susceptible to sun-induced skin cancer than less pigmented races [7-9]. This difference between races can be understood in terms of UV transmission of the epidermis [10]. The epidermis of black skin has more and larger melanosomes than that of white skin [11]. The absorption of light by melanin is nonspecific and extends through the UV into the visible ranges, but it is most pronounced toward the shorter end of the spectrum [7,8]. These facts suggest that epidermal melanin pigment prevents the development of UV-induced skin cancer by reducing UV transmission to underlying cell nuclei and by inhibiting the formation of UV-induced DNA damage.

Gange *et al* [12] found that a UV-induced tan reduced the yield of cyclobutane pyrimidine dimers in epidermal DNA. Tanning, however, tends to induce various epidermal changes including epidermal thickening, in addition to melanization. Thus, this system may not be proper for demonstrating the effect of melanin itself. Instead, cultured cells, containing different amounts of melanin, may be the most suitable system for determining the protective effect of melanin. Hill and Setlow [13,14] found that a lightly melanotic cell line formed fewer UV-induced cyclobutane dimers than a nonpigmented cell line. At the same time, they found that a more heavily melanotic cell line was not always more efficiently protected than a less melanotic cell line. Many investigators have also failed to find a protective effect of melanin on DNA using cells with different levels of melanin [15-19]. Thus, the protective effect of melanin on UV-induced DNA damage has not been fully proved experimentally, despite the UV-absorbing nature of melanin.

There are two possible explanations for these inconclusive results. First, there might be differences in the type of melanin, or in the size, shape, and localization of melanosomes among cell lines. These factors, in addition to melanin concentration, could possibly affect the photoprotection by melanin [10,11]. Second, there is a possibility that the methods were not sensitive enough to discriminate among the small differences in DNA damage formation. Thus, choice of a method with proper sensitivity must be taken into consideration. Furthermore, published papers have detected only cyclobutane dimers as UV-induced DNA damage, even though (6-4)photoproducts, in addition to cyclobutane dimers, have been known to be present in UV-irradiated DNA [5,20]. Recent evidence suggests that both photolesions are responsible for UV-induced cell killing [20,21] and mutations [22,23] in mammalian cells. Thus, we have established monoclonal antibodies specific for either cyclobutane dimers or (6-4)photoproducts [24-28]. The enzyme-linked immunosorbent assay (ELISA) method using these antibodies has made it possible to detect two types of photolesions simultaneously. The method was also found to be sensitive enough to measure both photolesions induced by low doses of UV radiation.

In this study, we have used three human melanoma cell lines containing different amounts of melanin. We investigated concentration, absorption spectrum, and subcellular localization of melanin in melanoma cells. Then, two types of photolesions were deter-

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Abbreviations: PBS-T, phosphate buffered saline at pH 7.4 containing 0.05% Tween 20; UPF, UV-protection factor.

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mined using the ELISA method. We also examined UV dose-survival curves using the colony-formation method. We found that intracellular melanin reduced the formation of UV-induced DNA damage and the cell killing rate in a melanin concentration-dependent manner.

MATERIALS AND METHODS

Cells and Media Three human malignant melanoma cell lines with different pigment levels were used. HM3KO, a heavily melanotic cell line, was a generous gift from Dr. M. Ichihashi (Kobe University) [29]. G-361 and Mewo, which were obtained from Japanese Cancer Research Resources Bank, are moderately and slightly melanotic cell lines, respectively. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Nissui Seiyaku, Tokyo) supplemented with 10% fetal bovine serum (FBS; Flow Laboratories, Inc., McLean, VA).

Determination of Cell Diameter Cells were harvested by trypsinization and photographed on a hemocytometer. Calipers were used to measure the largest and smallest diameters of photographs of each cell in more than 75 cells. The mean diameters of the cells were calculated by considering the magnification of the pictures.

Melanin Content in Cells Cells were harvested, counted by a hemocytometer, washed with 10 mM phosphate buffered saline (PBS) at pH 7.4, and centrifuged. The cell pellet was dissolved in 1 N sodium hydroxide [17]. Absorbance of intracellular melanin was measured at 400 nm with a UV-160A spectrophotometer (Shimadzu, Kyoto). The melanin content in the cells was then determined by comparing intracellular melanin absorbance with standard absorbance curves for synthetic melanin (Sigma).

Absorption Spectrum of Intracellular Melanin The melanosomal fraction from cells was extracted by the method of Seiji *et al* [30] with a minor modification. Briefly, cells were harvested, washed with PBS, and suspended in a 0.25-M sucrose solution. All the following procedures were carried out at 4°C. Cells were homogenized for 2 min in a Potter-Elvehjem-type homogenizer and centrifuged at $700 \times g$ for 10 min. The supernatant was then centrifuged at $11,000 \times g$ for 10 min, and the sediment was recentrifuged at $15,000 \times g$ for 10 min. Finally, this sediment, containing melanosomes, was again suspended in a 0.25-M sucrose solution. Absorbance of the melanosomal fraction extracted from 10^6 cells in 1 ml solution was measured in a range from 200 to 340 nm.

Localization of Intracellular Melanin To examine subcellular localization of melanosomes, we cultured cells on Celldesk (Sumitomo Bakelite, Tokyo), and fixed and embedded them in paraffin. Melanosomes in vertical sections were stained using Fontana-Masson method [31] and examined by a light microscope.

UV Irradiation and DNA Isolation The DNA isolation method has been described previously [28]. In brief, cells were plated in two 10-cm dishes (Falcon 3003) at a density of 2×10^6 per dish and cultured for 2 d. Cells were washed with PBS and UV-irradiated with two low-pressure mercury lamps (Toshiba GL-10, Tokyo; predominantly 254 nm UV) at a dose rate of 0.84 J/m^2/sec, which was monitored by a Topcon UV Radiometer (Topcon, Tokyo). Immediately after irradiation or after post-UV incubation for DNA repair, cells were lysed in 1 ml of 1% sodium dodecyl sulfate (SDS)-STE (100 mM NaCl, 50 mM Tris-HCl, 50 mM ethylenediamine tetraacetic acid [EDTA], pH 8.0) per dish. The lysates were digested with 200 μg/ml of Proteinase K for 16 h at 37°C. DNA, which had been extracted by the phenol/isoamyl alcohol-chloroform procedure, was dialyzed overnight against PBS at 4°C, and treated with 50 μg/ml of RNase A at 37°C for 1 h. After deproteinization, cellular DNA was dialyzed against PBS. Despite these extensive DNA purification procedures, we found a small quantity of melanin left in the DNA solutions from the three cell lines. Efforts to completely exclude melanin from the DNA solutions were unsuccessful. Therefore, it was decided to prepare DNA solutions containing the same quantity of melanin (equal to the heaviest mèlanized sample) by adding the purified melanin solution obtained from the melanosomal fraction. DNA does not have any absorption at 340 nm. Thus, the melanin amount in the DNA solution was calculated from the absorbance at this wavelength using the absorption curves of the purified melanin solution. DNA concentration was calculated from the absorbance at 260 nm after subtracting the melanin's absorbance.

DNA Damage Detection by ELISA The induction and repair of cyclobutane pyrimidine dimers and (6-4)photoproducts in UV-irradiated cells were measured by ELISA with monoclonal antibodies (TDM-2 for cyclobutane dimers and 64M-2 for [6-4]photoproducts). Details of the method have been described previously [28]. In brief, 96-well polyvinylchloride flat-bottom microtiter plates, pre-coated with 1% protamine sulfate, were incubated with DNA (15 ng/well for cyclobutane dimers and 300 ng/well for [6-4]photoproducts), extracted from UV-irradiated cells, for 20 h at 37°C. The plates were washed five times with PBS containing 0.05 % Tween 20 (PBS-T), and then incubated with 2% fetal bovine serum (FBS) for 30 min to prevent non-specific binding of the antibodies and washed again. One hundred microliters of the monoclonal antibody were added to the wells (in quadruplicate) and incubated at 37°C for 30 min. The plates were washed five times with PBS-T and then incubated with 100 μl of goat anti-mouse immunoglobulin G (H+L) conjugated with biotin, F(ab')$_2$ fragment (1/2,000 in PBS; Zymed, San Francisco, CA) for 30 min. After five washings with PBS-T, the plates were incubated with 100 μl of streptavidin conjugated with peroxidase (1/10,000 in PBS; Zymed) at 37°C for 30 min. Finally, after five washings with PBS-T and a subsequent washing with citrate-phosphate buffer (pH 5.0), 100 μl of substrate solution consisting of 0.4 mg/ml *o*-phenylene diamine and 0.02% H_2O_2 in citrate-phosphate buffer was added to each well. Following a 30-min incubation period at 37°C, 50 μl of 2 M H_2SO_4 was added to stop the reaction and absorbance at 492 nm was measured by Titertek Multiskan Plus MKII (Labsystems, Helsinki, Finland).

UV Survival Cells were plated in 10-cm dishes at densities of 1×10^3 (HM3KO) and 2×10^3 (G-361 and Mewo) per dish and cultured for 4 h. The culture medium was then removed and cells were UV irradiated in the same manner as previously described and cultured for 3 weeks. Colonies were then fixed, stained, and counted.

Table I. Melanin Concentration in Three Melanoma Cell Lines[a]

Cells	HM3KO	G-361	Mewo
Melanin content (pg/cell)	26.0 ± 1.1	5.5 ± 0.5	0.8 ± 0.2
Cell diameter (μm)	21.2 ± 3.4	20.0 ± 3.8	13.9 ± 2.7
Melanin concentration (μg/mm^3)	5.2 ± 0.2	1.3 ± 0.1	0.6 ± 0.2

[a] Each value shows the mean ± standard deviation (SD).

RESULTS

Determination of Melanin Concentration in Melanoma Cells Cell pellets of HM3KO, G-361, and Mewo were visually black, brown, and white, respectively. Melanin concentration was calculated using the melanin content per cell and the cell diameter. Table I summarizes the data. Highly pigmented HM3KO had the highest melanin concentration, followed by less pigmented G-361 and Mewo. Thus, the melanin levels in three types of melanoma cells were compatible with those expected from the pellet's color.

Absorption Spectrum of Intracellular Melanin We compared the absorption spectra of melanosomal fractions extracted from three types of melanoma cells. In this experiment, the absorbance may include scattering in addition to true absorption, because melanosomal fractions may contain various sizes and shapes of melanosomes. Figure 1 shows typical data of the absorbance from 200 to 340 nm. The absorption spectrum in each cell line showed a similar pattern. There was broad and featureless absorption increasing toward the shorter wavelengths with a peak near 200 nm. Thus, these three cell lines contained melanin with similar absorption spectra, but with different values of absorbance.

Localization of Intracellular Melanin To compare the subcellular localization of melanin with respect to the cell nucleus in three types of melanoma cells, we stained melanosomes in vertical sections of cultured cells using the Fontana-Masson method (Fig 2). The three cell lines showed similar localization with different amounts of intracellular melanin. Black granules (melanosomes) were observed to be diffusely located in whole cytoplasm and did not form the supranuclear caps observed in skin epidermis. The three cell lines had a flattened morphology, with minimal cytoplasm overlying the nucleus. Thus, black granules were located primarily on the sides of the nucleus rather than over it in the path of the UV irradiation. It was obvious, however, that highly pigmented HM3KO cells had the largest amount of black granules over the

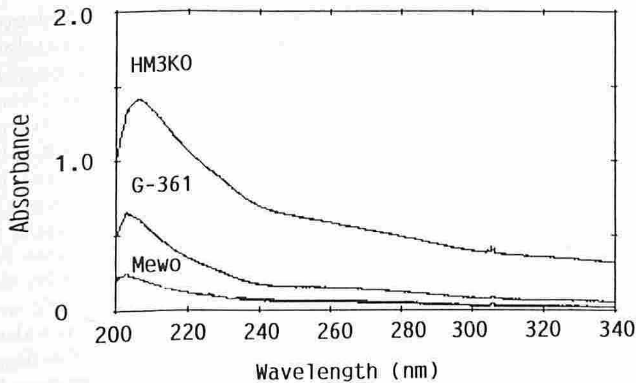

Figure 1. Absorption spectra of melanosomal fractions from human melanoma cells containing different amounts of melanin. A typical result from three independent experiments is shown.

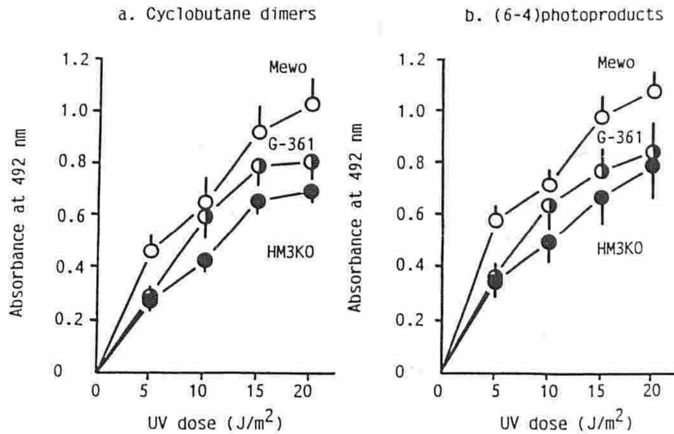

Figure 3. The induction of cyclobutane pyrimidine dimers *(a)* and (6-4)photoproducts *(b)* in DNA from UV-irradiated human melanoma cells containing different amounts of melanin. DNA damage was measured by the ELISA with monoclonal antibodies against photolesions. Each *point* shows the mean (± standard deviation, SD) of six determinations.

nucleus, followed by less pigmented G-361 and Mewo, respectively.

UV-Induced DNA Damage Formation in Melanoma Cells To examine the effect of melanin on UV-induced DNA damage formation, the induction of two types of photolesions in UV-irradiated melanoma cells with different pigment levels was detected by ELISA (Figs 3*a,b*). We found that the ELISA data were not affected by the presence of a small amount of melanin in the DNA solution (data not shown). Thus, we prepared DNA sample solutions containing equal, relatively small quantities of melanin, and detected DNA damage. The induction of cyclobutane dimers in the three cell lines increased in a UV dose-dependent manner. Heavily melanotic HM3KO cells had the lowest induction curve, whereas Mewo cells, with the lowest melanin concentration, showed the highest induction curve. Similar induction curves of (6-4)photoproducts were obtained in three UV-irradiated melanoma cell lines. More heavily melanotic cell lines had lower induction curves than less melanotic cell lines. These results suggest that melanin in melanoma cells reduces the formation of UV-induced DNA damage (both cyclobutane dimers and [6-4]photoproducts) in a melanin concentration-dependent manner.

DNA Repair in Melanoma Cells We compared the repair capacity for cyclobutane pyrimidine dimers and (6-4)photoproducts in three types of melanoma cells (Figs 4*a,b*). HM3KO, G-361, and Mewo cells were UV irradiated at 20, 15, and 10 J/m^2, respectively, to induce similar numbers of photolesions, and then incubated for DNA repair. The percentage of the initial number of both photolesions was determined at various times after UV irradiation. No significant differences in the repair capacity for both photolesions were observed among the three melanoma cell lines.

UV-Induced Cell Killing in Melanoma Cells To evaluate the effect of melanin on UV-induced cell killing, we determined survival after UV irradiation in three melanoma cell lines by the colony-formation method (Fig 5). Heavily melanotic HM3KO cells were the most UV resistant, followed by G-361 and Mewo cells. Thus, more melanotic cell lines were more UV resistant than less melanotic cell lines. This result suggests that the presence of melanin in cells reduces the UV-induced cell killing rate in a concentration-dependent manner.

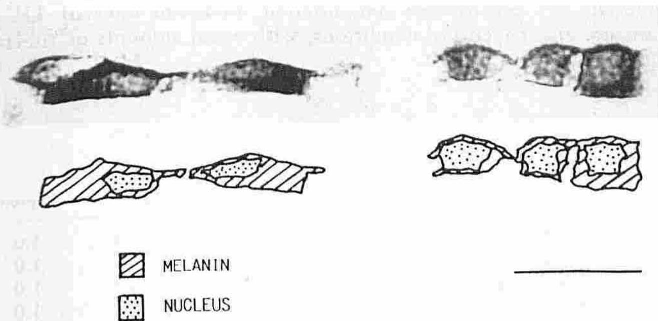

Figure 2. Subcellular localization of melanin in cultured melanoma cells. Melanosomes in vertical sections of cultured cells were stained with the Fontana-Masson method. The three cell lines showed similar localization with different amounts of intracellular melanin. A picture of HM3KO cells *(upper)* and its schema *(lower)* are shown. *Bar,* 50 μm.

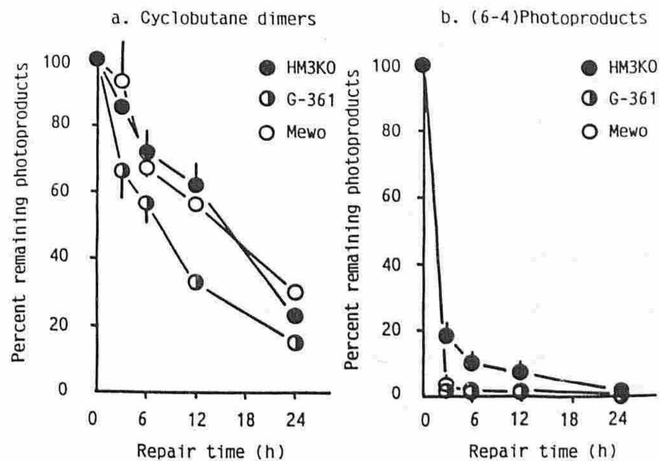

Figure 4. The repair capacity for cyclobutane pyrimidine dimers *(a)* and (6-4)photoproducts *(b)* in melanoma cells. HM3KO, G-361, and Mewo cells were UV irradiated at 20, 15, and 10 J/m^2, respectively, to induce similar numbers of photolesions. The percentage of the initial number of photolesions was determined at various times after UV irradiation. Each *point* shows the mean (± SD) of four determinations.

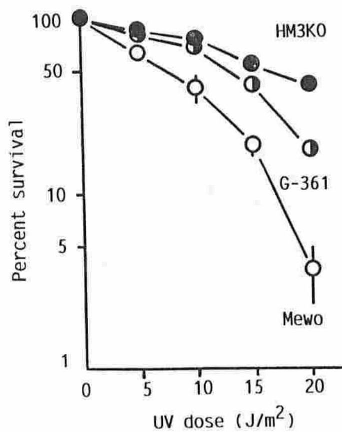

Figure 5. The UV survival curves of cultured human melanoma cells containing different amounts of melanin. Each *point* shows the mean (± SD) of three independent experiments.

DISCUSSION

We took many precautions in examining the effect of melanin on UV-induced DNA damage. First, cultured cells, not skin epidermis, were used, and three human melanoma cell lines containing different amounts of melanin were selected. The relation of the position of melanin to the cell nucleus for protection against UV irradiation is simpler in cultured melanoma cells than in multilayered skin epidermis. This simplicity is beneficial to evaluating the protective effect of melanin itself. The melanin concentration was determined in the three cell lines. There was an almost ninefold difference between the least and most pigmented cells (Table I). Furthermore, we made certain that the three melanoma cell lines contained melanin with similar absorption patterns between 200 and 340 nm with different values of absorbance (Fig 1). These absorption spectra are typical for melanin [7,8] and suggest that melanin in these cell lines has similar ability to reduce UV-induced damage. Moreover, the three cell lines had similar subcellular localization with different amounts of melanin (Fig 2). Melanin was observed to be diffusely located in whole cytoplasm, and there was minimal cytoplasm overlying the nucleus because of the flattened morphology of the cultured melanoma cells. Thus, melanin was located primarily on the sides of the nucleus rather than over it, in the path of the UV irradiation. This suggests that intracellular melanin in cultured cells may not shield the nucleus as effectively as supranuclear melanin caps observed in epidermal keratinocytes. However, it was apparent microscopically that highly pigmented HM3KO cells had the largest amount of melanin over the nucleus, followed by less pigmented G-361 and Mewo cells.

Second, we used one of the most sensitive detection systems for DNA damage. The ELISA with monoclonal antibodies against photolesions made it possible to detect two types of DNA damage, cyclobutane pyrimidine dimers and (6-4)photoproducts, induced by low UV doses. Similar levels of UV doses were also used for examining UV-induced cell killing by the colony-formation method. We found that intracellular melanin reduced the formation of both types of DNA damage in UV-irradiated melanoma cells in a melanin concentration-dependent manner (Figs 3*a,b*). We also found that intracellular melanin protected melanoma cells from UV-induced cell killing in a melanin concentration-dependent manner (Fig 5). We calculated UV-protection factors (UPFs) to compare the induction rates of photolesions and the killing rates among the three types of melanoma cells (Table II). It was observed, however, that UPF values varied depending on the criteria for calculation, as both induction curves for photolesions and survival curves were not linear. Thus, we decided to adopt the criteria shown in Table II to roughly compare the induction rates and killing rates among the three melanoma cell lines. Similar UPFs in three melanoma cell lines were obtained from the induction rates of two types of photolesions. This result suggests that melanin shields the cell nucleus from UV and reduces the formation of two types of DNA damage in the same way. Furthermore, there was a good correlation between UPFs for percent survival and for two types of photolesions among three melanoma cell lines. This result suggests that intracellular melanin protects melanoma cells from UV-induced cell killing by reducing the formation of two types of DNA damage. This hypothesis is supported by the results from the DNA repair experiment in which almost no differences were observed in the repair capacity for both photolesions among the three cell lines (Figs 4*a,b*). On the other hand, relative melanin concentration among the three cell lines was also calculated using data in Table I (Table II). This value, however, did not match well with UPFs obtained from DNA damage formation and cell killing. The most likely explanation may be that the melanin concentration in each cell line does not reflect the actual amount of melanin responsible for shielding the nucleus from UV irradiation. As previously mentioned, only a small amount of melanin is located over the nucleus in the path of the UV irradiation. Thus, the relative amounts of melanin over the nucleus in three cell lines might be compatible with the UPFs, although this could not be verified in the present experiment.

There have been two papers reporting the protective effect of melanin on formation of UV-induced cyclobutane dimers. They found UPF values (1.8 – 2.2) similar to those obtained in the present study [12,32]. On the other hand, others could not find a protective effect of melanin on formation of UV-induced cyclobutane dimers [15 – 19]. We can not account for the differences between these contradictory results. One possibility might be that intracellular melanin could not efficiently shield the nucleus against UV irradiation because of the inadequate subcellular localization for shielding. No published studies, however, have examined the subcellular localization of melanin. Another possibility might be that DNA damage detection systems were not sensitive enough to discriminate among the small differences in DNA damage induced in the cells. We believe the ELISA method to be more sensitive than systems such as chromatographic analysis or unscheduled DNA synthesis. In this experiment, we have shown that the ELISA method possessed the sensitivity required for detecting two types of photolesions induced by low UV doses.

In this study, we found that intracellular melanin protected cultured melanoma cells from UV-induced DNA damage formation and cell killing in a concentration-dependent manner. Hacham *et al* [33] have suggested that endogenous pigmentation reduces the rate of induction of cyclobutane dimers in the DNA of human skin irradiated *in situ* with UV light (275 – 365 nm). From their results and ours, we can suggest that epidermal melanin pigment plays an important role in preventing UV-induced skin cancer by reducing DNA damage and cell killing, because photocarcinogenesis is a multistep process involving initiation, promotion, and progression [6,34]. However, the three human melanoma cell lines used in the present study may have different genetic backgrounds (possibly, different UV sensitivities and different induction rates of UV-damage, under identical conditions, with equal amounts of mela-

Table II. UV Protection Factors (UPFs) in Three Melanoma Cell Lines

Cells	HM3KO	G-361	Mewo
UPFs for cyclobutane dimers[a]	2.4 ± 1.3	1.6 ± 0.5	1.0
UPFs for (6-4)photoproducts[a]	2.3 ± 0.5	1.7 ± 0.1	1.0
UPFs for percent survival[b]	2.2 ± 0.3	1.7 ± 0.3	1.0
Relative melanin concentration[c]	8.7 ± 0.4	2.2 ± 0.2	1.0

[a] Calculated from UV doses showing 0.5 of absorbance at 492 nm in Figs 3*a,b* (mean ± SD). The value of Mewo was expressed as 1.0.

[b] Calculated from UV doses showing 50% survival in Fig 5 (mean ± SD). The value of Mewo was expressed as 1.0.

[c] Calculated from melanin concentration in Table I (mean ± SD). The value of Mewo was expressed as 1.0.

nin). To totally exlude these uncertainties, similar experiments are in progress using human melanoma cells with the same genetic background irradiated with UV-C and UV-B light.

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