Photocarcinogenesis in human adult skin grafts

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It has been demonstrated previously that the exposure to 7,12-dimethyl[a]benzanthracene (DMBA) and UVB radiation leads to the development of epidermal cysts, squamous cell carcinomas (SCC), melanocytic hyperplasia and melanoma in human foreskins from newborns grafted to immunodeficient mice. Improved techniques in grafting full-thickness skin from adults have enabled us to study photocarcinogenesis in human skin from different body sites and from older donors. One hundred and fifty-five normal white skin specimens from the trunk and face of 53 adult individuals were grafted onto severe combined immunodeficient (SCID) and recombinase activating gene-1 (Rag-1) knockout mice and irradiated two to three times weekly with 40 mJ/cm² UVB or solar-simulated UV (SSUV) over a period of up to 10 months with or without one prior topical application of DMBA. Over an observation period of 2–22 months, histopathological and immunohistochemical analyses of 134 specimens revealed actinic keratoses in 30% of the DMBA- + UVB-treated grafts, in 18% of the grafts exposed to SSUV only, and in 10% of the grafts exposed to UVB only. Actinic keratoses were absent in grafts treated with DMBA only. One SCC was found in an abdominal skin graft 3 months after exposure to DMBA followed by UVB. Point mutations in codon 61 of the human Ha-ras gene were detected in the SCC, five of six analyzed actinic keratoses and in non-lesional epidermis of DMBA- and UVB-treated grafts, indicating that DMBA as well as UVB alone can induce these mutations in human skin. In contrast to the previous experience with neonatal foreskin grafts, melanocytic lesions were not found except for mild hyperplasia in few cases. The data suggest that melanocytes from young individuals are more susceptible to the transforming effects of genotoxic agents than melanocytes from adults.

Introduction

The most prominent environmental risk factor for the development of skin cancer in humans is exposure to sunlight, which has been documented by epidemiological data (1,2). While chronic cumulative exposure to UV radiation of the sun has been associated with non-melanoma skin cancer, intense intermittent exposure has been associated with melanoma (3). In mice, it has been shown that UVB is capable of initiating skin carcinogenesis leading to squamous cell carcinoma (SCC) and its precursor lesions (4). The role of UVA, which is found much more abundantly in the sunlight, is less clear; however, its oxidative DNA-damaging as well as immunosuppressive effects are believed to contribute to skin cancer development (5).

Mouse models of two-stage skin carcinogenesis have been developed, in which 7,12-dimethyl[a]benzanthracene (DMBA) is used as the initiator and 12-O-tetradecanoylphorbol-13-acetate (TPA) or UV radiation as the tumor promoter (6). DMBA has been shown to induce an activating point mutation (A→T) at the second position of codon 61 of the Ha-ras gene and much evidence indicates that such activating Ha-ras mutations serve as initiating events in mouse skin tumorigenesis (7–12). In humans, Ha-ras codon 61 mutations have been found by different investigators in 0–20% of non-melanoma skin cancers or benign skin neoplasms, and UV radiation has been discussed as the possible cause (13–17). Our laboratory has recently developed a human photocarcinogenesis model, in which human skin grafted to immunodeficient mice is topically treated with DMBA once and subsequently irradiated with UVB over several months (18,19). In this model, human SCC and its precursor lesions develop and we now provide evidence that the Ha-ras codon 61 point mutation can be induced in human skin by DMBA or UV radiation alone.

While progress has been made in understanding the development of non-melanoma skin cancers, in which UV-mediated immunosuppressive effects and UV-induced mutations in the p53 and perhaps ras genes through DNA damage at dipyrimidine sites have been associated with photocarcinogenesis (20,21), little is known about the biological and molecular events in the UV-associated development of melanoma, in which p53 mutations, for example, do not seem to play a major role. UV induction of melanoma could be demonstrated in Xiphophorus hybrid fishes and in opossums (22,23). These studies have suggested that UVA may play a role in melanoma development different from that described for UVB in non-melanoma skin cancers. However, these findings are difficult to apply to humans because of genetic and anatomical differences. Recently, we have shown that in human foreskins from newborns grafted to immunodeficient mice, exposure to UVB can lead to the development of melanocytic hyperplasia and, when combined with DMBA, to melanoma (24). This was the first report on UV-promoted melanoma formation in human skin. As these studies were carried out with neonatal foreskins representing an uncommon site and age for melanoma develop-

Abbreviations: AK, actinic keratosis; DMBA, 7,12-dimethyl[a]benzanthracene; H&E, hematoxylin and eosin; MED, minimal erythematous dose; MSPA, mutation-specific PCR analysis; Rag, recombinase activating gene; SCC, squamous cell carcinoma; SCID, severe combined immunodeficient; SSUV, solar-simulated UV.
ment in humans, we have now analyzed skins from different body sites and from older donors. White-colored adult skin could be selected for grafting, whereas the foreskin specimens often appeared light in the beginning, but then became tan or dark brown at later time points. Besides UVB light, solar-simulated UV (SSUV) was used in a subgroup to include the potentially deleterious effects of UVA. While non-melanoma skin cancer lesions developed at similar rates as described before, melanocytic changes were much rarer in the adult skin grafts in all groups when compared with the foreskin grafts from newborns analyzed previously. The data suggest that melanocytes from young individuals are more likely to be transformed by UV light than melanocytes from older individuals and may explain why sunburn in childhood is of higher risk for melanoma development than in adults.

Materials and methods

Human skin grafting

Normal human skin specimens were obtained from adult donors who underwent plastic surgery of their abdomen (n = 15), breast (n = 10) or face (n = 13) (Cooperative Human Tissue Network, Philadelphia, PA), or who donated skin from their back or buttocks (n = 4), and from patients who underwent wide excision of cutaneous melanoma (n = 11) (Department of Dermatology, University of Pennsylvania, Philadelphia, PA). Approval by the Institutional Review Board was obtained for acquisition and use of human tissue in all experiments. Specimens were kept in sterile transport media (RPMI-1640 or Hank’s balanced salt solution supplemented with antibiotics) and grafted within 48 h of excision as described (25). The median number of grafts per specimen was 2 (range 1–10). Female and male C.B-17 severe combined immunodeficient (SCID) mice (bred at the Animal Facility of the Wistar Institute) and B6,129S-Rag-1 tm1Mom mice (Jackson Laboratory, Bar Harbor, ME) were housed under pathogen-free conditions in groups of up to five animals per isolator cage. At 6–10 weeks of age, a 1–3 cm² skin segment behind the shoulder of the animal was excised, leaving the panniculus carnosus muscle intact. The wound was immediately covered with full-thickness human skin that was held in place by the bandage alone or by 6–0 non-absorbable poliviolene sutures. The bandage consisted of non-adhesive Vaseline dressing, sterile sponges and surgical tape, and was changed after 2 weeks. Grafts were well healed after 4–6 weeks and used for the experiments. The Wistar Institutional Animal Care and Use Committee approved all protocols and the facility is approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

UV irradiation

UVB light was provided by two Westinghouse FS72T12/UVB lamps (UV Resources International, Lakewood, OH) with an output range of 280–370 nm and a peak at 313 nm. The light was filtered through cellulose triacetate Kodacel TA 407 sheets (Eastman Kodak, Rochester, NY) to exclude wavelengths below 295 nm. The UV dose was continuously monitored with a PMA 2100 radiometer (Solar Light, Philadelphia, PA) and ranged between 30 and 50 mJ/cm² for UVB and 0.1 and 0.2 J/cm² for UVSSUV light was provided by two UVA 340 lamps (Q-Panel, Cleveland, OH) with an output range of 295–400 nm and a peak at 340 nm. The irradiation dose was 30 mJ/cm² UVB and 3–6 J/cm² UVA. The wavelength spectra of both lamps were measured and analyzed at Argus Research Laboratories (Philadelphia, PA) by the kind support of Dr Donald Forbes. During irradiations, mice were separated from each other and allowed to move freely in the cage. Irradiations were performed two to three times weekly for 10–30 min each time over a period of 2–10 months.

Treatment groups

The experimental protocol and the five different treatment groups are outlined in Figure 1. When several grafts were derived from the same donor, they were distributed into different groups. DMBA (Sigma, St Louis, MO) was dissolved in acetone and applied once onto the skin grafts at a concentration of 15 µg/cm². DMBA treatment was performed 1–25 days before the UV irradiations.

Histology and immunohistochemistry

Mice were killed by CO₂ inhalation and skin grafts were excised. Half of the grafts were fixed in 10% neutral-buffered formalin (Fisher Scientific, Pittsburgh, PA) for 6–12 h at room temperature and embedded in paraffin. The other half was dehydrated by increasing concentrations of sucrose solutions (5, 10 and 20%) at 4°C overnight, embedded in OCT medium.

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**Fig. 1.** Experimental protocol and treatment groups of photocarcinogenesis study. MED, minimal erythematous dose; SSUV, solar-simulated UV.

(Miles, Elkhart, IN), snap-frozen and stored at −70°C until cryosectioning. Formalin-fixed sections were stained with hematoxylin and eosin (H&E) for histopathological evaluation. The DNA-binding fluorochrome Hoechst 33258 (Sigma, St Louis, MO) was used to distinguish human from murine cells (26). Immunohistochemistry was performed on serial sections using an avidin–biotin–peroxidase system kit (Vector Laboratories, Burlingame, CA) and 3,3’-diaminobenzidine tetrahydrochloride (Sigma) or 3-amin-9-ethylcarbazole (Vector) as chromogens. Antigens in the formalin-fixed tissues were retrieved by trypsin digestion at 37°C or microwave heat treatment in citrate buffer. Cryostat sections of 6–8 µm were air-dried and fixed in ice-cold acetone for 10 min. Before incubation with the primary antibodies in a humidified chamber at 4°C overnight or at room temperature for 1–2 h, non-specific binding was blocked with 10% normal horse or 10% normal goat serum. Primary mouse monoclonal antibodies used in this study were: anti-human Ki-67 (clone MIB-1, IgG1, Immunotech, Westbrook, ME); anti-human pancytokeratin (clone AE1/AE3, IgG1, Zymed, San Francisco, CA); anti-human TRP-1/fgp75 (clone T99, IgG2a, kind gift from Dr V. Setaluri, Winston-Salem, NC); and anti-human HMB45 (IgG1, Biogenex, San Ramon, CA). A polyclonal rabbit antibody was used for detection of S100 (Dako, Carpinteria, CA). A mouse IgG1 isotype antibody (P3×63Ag8) was used as negative control for each staining. Between each incubation step, slides were rinsed twice in phosphate-buffered saline for 3–5 min. A biotin-labeled anti-mouse or anti-rabbit secondary antibody was applied for 30 min at room temperature followed by incubation with a preformed avidin–biotinylated enzyme complex for 30 min. After color development by addition of the chromogen and counterstaining with Mayer’s hematoxylin (Sigma), sections were mounted and evaluated under a light microscope.

**Ha-ras mutation analysis**

Mutations in the second position of codon 61 (A→T) of the human Ha-ras gene were detected using the mutation-specific PCR assay (MSPA) as described previously for mice (27) with modifications. The assay depends on the use of 3′ primers specific for either the wild-type or mutant sequences. Because of sequence differences, the primers used for analysis of the human Ha-ras gene do not yield any specific products with murine DNA. Briefly, DNA for MSPA was prepared from samples microdissected from unstained sections of paraffin-embedded, formalin-fixed tissue, using H&E-stained sections for reference. DNA from normal human placenta (Sigma) and a human tumor cell line (CRL 5904, ATCC, Manassas, VA), known to be positive for the Ha-ras codon 61 mutation, were included as negative and positive controls, respectively. Additionally, DNA from normal mouse spleen and a mouse carcinoma known to be positive for the Ha-ras codon 61 mutation were included as controls to verify species specificity. Human Ha-ras primers were obtained from Sigma Genosys (The Woodlands, TX). The primers used for the MSPA amplification were as follows: 5′ primer, 5′-TGAGCCCTGTCCTCCTGCAG-3′; 3′ wild-type primer, 5′-ATGGCGCTGTACTCCTCCA-3′; and 3′ mutant primer, 5′-ATGGCGCTGTACTCTCCCA-3′. These primers generate either a 110 (wild-type) or 109 bp (mutant) product. An internal oligonucleotide probe specific for the human Ha-ras PCR product was also synthesized.
lesions was confirmed by nuclear Hoechst staining and detection of the human-specific proliferation marker Ki-67 in the epidermis and in the tumors. An example of a DMBA + UVB-treated abdominal skin graft, which did not develop skin lesions within 13 months of observation, is shown in Figure 2F and G. Hyperkeratosis, thickening of the epidermis (acanthosis), and elastosis in the dermis were commonly found in the skin grafts in all groups. However, these responses were excluded from our comparative analysis, because they were also commonly found in untreated grafts when they were compared with the ungrafted donor skin. A histological comparison between foreskin grafts from newborns and skin grafts from adults revealed that hyperkeratosis and epidermal thickening developed in both types of skin after grafting to the mouse. However, elastosis was only found in the adult skin grafts.

Both the exposure to DMBA and the exposure to UV-induced carcinogenesis in the mouse at a higher frequency than in the human skin. This was the case even though DMBA was only applied onto the human skin graft. In all treatment groups, AK and SCC of murine origin developed adjacent to the human skin grafts in 8–29% and 8–35%, respectively (Table II). The highest incidences of murine AK and SCC were found in the group exposed to SSUV only. In addition, a few poorly differentiated malignant tumors in the eyes as well as AK and SCC on the ears and tails of UV-irradiated mice were observed (not shown).

Development of melanocytic lesions
Increased pigmentation due to the UV irradiation was observed in some grafts, while others did not tan, but repeatedly developed slight erythema 24 h after irradiation. No grossly visible pigmented lesions developed in any of the grafts. At the microscopic level, one graft irradiated with UVB only, and two grafts treated with DMBA + UVB, showed a slight hyperplasia of single melanocytes. In another UVB-treated graft, melanocytic hyperplasia with beginning nest formation was found (Table I and Figure 2H). In other skin grafts melanocytes were normal or appeared to be diminished.

Ha-ras mutations in human skin grafts
A total of 25 samples were analyzed for point mutations (A→T transversions) in the second position of codon 61 of the human Ha-ras gene by MSPA. These included specimens microdissected from 13 human skin grafts from eight different donors, and as controls, microdissected epidermis from nontreated skin grafts and donor skin before grafting. The samples analyzed and results are summarized in Table III. Four specimens did not yield PCR products with either the...
wild-type- or mutation-specific primers and were considered non-informative (not included in Table III). The untreated grafts and donor skin were all negative for the point mutation in codon 61 of the human Ha-ras gene. The SCC, five of six analyzed AK, two areas with melanocytic hyperplasia, and non-lesional epidermis from DMBA+, bFGF + UVB- and DMBA + UVB-treated grafts were positive for this mutation. The cutaneous lymphoma microdissected from the dermal area beneath the described SCC did not have the specific Ha-ras mutation.

Representative Southern blots of the PCR products from human Ha-ras gene MSPA are shown in Figure 3. The human wild-type- and mutation-specific primers did not yield PCR products with either normal mouse spleen DNA (Figure 3A and B, lanes 2 and 1, respectively) or with mouse carcinoma DNA carrying the specific A→T mutation in codon 61 of the murine Ha-ras gene (Figure 3A and B, lanes 3 and 2, respectively). A specificity control included in all analyses was human placental DNA, which consistently produced PCR products with the human wild-type-specific primers (Figure

Fig. 2. Photocarcinogenesis in human adult skin grafts. (A) Human AK on a non-pigmented face skin graft 4 months after beginning of treatment with DMBA once and subsequently with UVB three times weekly. (B) Histological sections of a human AK in a breast skin graft treated with DMBA and SSUV (H&E, ×100). (C) Immunohistochemical detection of Ki-67 in the same specimen as in B (×100). (D) Human SCC in a face skin graft treated with DMBA once and irradiated with UVB over 3 months. (E) Expression of cytokeratin in the same human SCC as in D (×400). (F) Breast skin graft 6 months after beginning of treatment with DMBA once and subsequent UVB irradiations. (G) H&E-stained section of the same graft as in F 7 months later (×100). (H) Melanocytic hyperplasia with beginning nest formation (arrows) in a face skin graft after 9 months of UVB irradiation.
3A, lane 4), but was consistently negative with the mutation-specific primers (Figure 3B, lane 3). The positive control for the mutation analysis was human tumor cell line CRL 5904, which is known to have the specific Ha-ras codon 61 mutation. These cells apparently have copies of both the normal and mutant Ha-ras gene. DNA from these cells consistently produced PCR products with both the wild-type- and mutation-specific primers (Figure 3A and B, lanes 5 and 4, respectively). Based on uniformly consistent results with the species- and sequence-specific controls, we conclude that mutations detected in grafts were not artifacts due to murine contamination or amplification of the wild-type sequence.

**Discussion**

In an in vivo model of photocarcinogenesis in human adult skin we have demonstrated the development of AK, a precursor lesion of SCC, and of epidermal cysts by UV radiation alone. The incidence of these UV-associated skin lesions was higher in skin grafts exposed to SSUV, i.e., UVB and UV A, than in skin grafts exposed to UVB only. This difference was even greater when exposure to UV was combined with DMBA, incidences of AK were not different between skin grafts irradiated with SSUV or with UVB only. Therefore, further investigations are needed to provide evidence for additive effects of UVB and UVA. The additional exposure to DMBA led to human SCC; however, the incidence of 2% in the DMBA-UVB-treated human skin grafts was relatively low. Aggressive growth of murine skin cancer into the human skin grafts, which often led to the premature termination of the experiment, may have inhibited the development of more human SCC over an extended period of time. The higher incidence of murine skin cancer lesions was probably due to the high susceptibility of murine cells to transformation, the thin epidermis in mice facilitating penetration of carcinogens, and the lack of protecting pigmentation in the albino SCID mice.

Mutations in codon 61 of the human Ha-ras gene were detected in both normal epidermis and in squamous and melanocytic lesions in skin grafts exposed to UVB, DMBA, or UVB + DMBA, whereas non-treated controls from the same donors before and after grafting did not have these mutations. The induction of mutation by UVB at codon 61 is consistent with the sequence of the human Ha-ras gene around codon 61 (Figure 3D). In the transcribed (anticoding) strand there is a run of pyrimidines beginning at the second position of codon 61. Tormanen and Pfeifer (29) reported mapping a high frequency of UV-induced cyclobutane dimers and (6–4) photoproducts in the transcribed strand of the Ha-ras gene near codon 61. The A→T transversion detected in the Ha-ras gene is not a signature UV radiation-induced mutation, because the incidences of murine AK and SCC, which developed adjacent to the human skin grafts, were compared. The data suggest an additive effect of UVB and UVA in the development of non-melanoma skin cancer, most likely, because they induce DNA damage via different routes, i.e., directly at dipyrimidine sites (UVB) and indirectly through oxidative damage (UVA). However, when exposure to UV was combined with DMBA, incidences of AK were not different between skin grafts irradiated with SSUV or with UVB only. Therefore, further investigations are needed to provide evidence for additive effects of UVB and UVA. The additional exposure to DMBA led to human SCC; however, the incidence of 2% in the DMBA-UVB-treated human skin grafts was relatively low. Aggressive growth of murine skin cancer into the human skin grafts, which often led to the premature termination of the experiment, may have inhibited the development of more human SCC over an extended period of time. The higher incidence of murine skin cancer lesions was probably due to the high susceptibility of murine cells to transformation, the thin epidermis in mice facilitating penetration of carcinogens, and the lack of protecting pigmentation in the albino SCID mice.

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### Table II. Murine skin cancer lesions adjacent to human adult skin grafts treated with DMBA, UV or a combination of both

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>n</th>
<th>Average months observed (range)</th>
<th>AK</th>
<th>SSC</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMBA</td>
<td>13</td>
<td>10 ± 4 (3–16)</td>
<td>1 (8%)</td>
<td>1 (8%)</td>
</tr>
<tr>
<td>SSUV</td>
<td>17</td>
<td>8 ± 5 (3–21)</td>
<td>5 (29%)</td>
<td>6 (35%)</td>
</tr>
<tr>
<td>UVB</td>
<td>24</td>
<td>11 ± 6 (3–22)</td>
<td>3 (13%)</td>
<td>6 (25%)</td>
</tr>
<tr>
<td>DMBA + SSUV</td>
<td>49</td>
<td>10 ± 4 (2–19)</td>
<td>8 (16%)</td>
<td>14 (29%)</td>
</tr>
</tbody>
</table>

*The average observation time was 9–10 months with a range of 2–22 months.

AK, actinic keratosis; SCC, squamous cell carcinoma; SSUV, solar-simulated UV.

### Table III. Ha-ras mutation analysis at codon 61

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Months observed</th>
<th>Body site</th>
<th>Diagnosis</th>
<th>Ha-ras wild-type</th>
<th>Ha-ras mutation</th>
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</thead>
<tbody>
<tr>
<td>DMBA</td>
<td>9</td>
<td>Abdomen</td>
<td>Normal epidermis</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>UVB</td>
<td>9</td>
<td>Face&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Melanocytic hyperplasia</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>Face&lt;sup&gt;b&lt;/sup&gt;</td>
<td>AK</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Face&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Normal epidermis</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Face&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Normal epidermis</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>Abdomen&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Normal epidermis</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Abdomen&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Melanocytic hyperplasia</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Abdomen&lt;sup&gt;g&lt;/sup&gt;</td>
<td>AK</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Face&lt;sup&gt;h&lt;/sup&gt;</td>
<td>SCC</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>3</td>
<td>Face&lt;sup&gt;i&lt;/sup&gt;</td>
<td>Cutaneous lymphoma</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Face&lt;sup&gt;j&lt;/sup&gt;</td>
<td>AK</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Abdomen&lt;sup&gt;k&lt;/sup&gt;</td>
<td>AK</td>
<td>+</td>
<td>+</td>
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<tr>
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<td>Abdomen&lt;sup&gt;l&lt;/sup&gt;</td>
<td>Normal epidermis</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>No treatment</td>
<td>12</td>
<td>Breast&lt;sup&gt;m&lt;/sup&gt;</td>
<td>Normal epidermis</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Before grafting</td>
<td>n/a</td>
<td>Face&lt;sup&gt;n&lt;/sup&gt;</td>
<td>Normal epidermis</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>n/a</td>
<td>Abdomen&lt;sup&gt;o&lt;/sup&gt;</td>
<td>Normal epidermis</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>n/a</td>
<td>Breast&lt;sup&gt;p&lt;/sup&gt;</td>
<td>Normal epidermis</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

AK, actinic keratosis; <sup>a,b,c,d,e</sup> = same donor, respectively; n/a, not applicable; SCC, squamous cell carcinoma. Mutations in the second position of codon 61 of the human Ha-ras gene (A→T) were analyzed in human skin grafted to immunodeficient mice and ungrafted donor skin. Grafts were either untreated or treated with DMBA + UVB, DMBA alone, UVB alone, or UVB + bFGF expressed in the skin by injection of adenoviral vectors for bFGF (28). Cells from lesions or normal epidermis were manually microdissected and analyzed by MSPA as described in Materials and methods.
Fig. 3. Analysis of the human Ha-ras gene for an A→T transversion in the second position of codon 61 by MSPA. (A–C) Products probed with an Ha-ras-specific 32P-labeled oligomer. Representative sample data and specificity controls are shown. (A) Products with wild-type PCR primers. Sources of DNA analyzed were as follows: 1, no template; 2, normal mouse spleen; 3, mouse carcinoma (C1977) known to be positive for the codon 61 mutation; 4, human placenta; 5, human tumor cell line CRL 5904 known to be positive for the codon 61 mutation; 6, ungrafted human breast donor skin. (B) Products with mutation-specific primers. Samples were as follows: 1, normal mouse spleen; 2, mouse carcinoma C1977; 3, human placenta; 4, human tumor cell line CRL 5904; 5, blank lane; 6, ungrafted human breast donor skin. (C) Analysis of samples from treated human skin grafts with mutation-specific primers. Samples were as follows: 1, SCC in facial skin induced by DMBA + UVB for 3 months; 2, cutaneous lymphoma from the same graft as in 1; 3, AK in facial skin graft treated with DMBA + UVB for 5 months; 4, mixed sample of hyperplastic melanocytes and keratinocytes in facial skin graft treated with UVB alone for 9 months; 5, normal epidermis from abdominal skin treated with bFGF + UVB for 2 months. The weak positive signal in lane 5 was confirmed on a separate gel and Southern blot. (D) Sequence of the human Ha-ras gene at codons 61–63. Note the run of pyrimidines beginning at the second position of codon 61 on the transcribed (anticoding) strand.

it can be induced by other agents. The induction of this mutation by DMBA may be the result of the formation of bulky adducts with adenine (30). Germicidal UV radiation has been shown to induce T→A transversions at dipyrimidine sites in bacteria and human cells, and runs of pyrimidines are mutational hotspots (31,32). This mutation induced in the transcribed strand yields the A→T mutation in the non-transcribed (coding) strand. The data presented here provide for the first time experimental evidence that activating mutations in the Ha-ras gene can be induced in human skin by DMBA and by UVB alone. It is possible that in a subset of non-melanoma skin cancers in humans, Ha-ras mutations are the initiating molecular event in UV-associated carcinogenesis.

Melnocytic changes were rare in the adult skin grafts in all treatment groups with an incidence of up to 6%. Compared with our experience with neonatal foreskin grafts, in which melanocytic hyperplasia occurred in 68% of the grafts treated with UVB only and in 77% of the grafts treated with DMBA and UVB (24), melanosocytes in adult skin seemed to be better protected from the genotoxic effects of UVB and DMBA. This may be due to the different proliferation and activation status of the melanosocytes in adults versus children. Whereas melanosocytes in the skin of adults are usually quiescent and divide only rarely, melanosocytes in growing individuals still have to divide frequently to account for the increasing body surface (33). Yet, in the state of proliferation, cells are commonly more susceptible for the acquisition of mutations, which, in the case of melanoma, may be induced by intense exposure to UV radiation of the sunlight (3). This transformation hypothesis is supported by our recent finding, that melanoma can develop in adult human skin grafts, when melanocytes are exposed to both the mitogen bFGF and to UVB, but not either of them alone (28). Our data, therefore, provide experimental support for the epidemiological observations that sunburn in childhood is associated with a higher risk for melanoma development than in adulthood (34,35).

In conclusion, we have shown the development of non-melanoma skin cancer and its precursor lesions in adult human skin exposed to UVB or SSUV and DMBA. Point mutations in the codon 61 of the human Ha-ras gene were found to be induced by either DMBA or UVB in human skin and may be the initiating event in a subset of UV-induced skin cancers in humans. The low incidence of experimental melanocyte transformation in human adult skin supports the concept of age dependence of melanoma induction, that potentially is highest in childhood and adolescence.

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