Induction of Melanoma Phenotypes in Human Skin by Growth Factors and Ultraviolet B

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Abstract

Exposure to UV radiation likely plays a key role in melanoma development, whereas other etiologic agents remain unknown. Here we show that in normal human skin an increased expression of a combination of three growth factors, basic fibroblast growth factor, stem cell factor, and endothelin-3, along with exposure to UVB can transform normal melanocytes into a melanoma phenotype within 4 weeks. Invasion of melanoma lesions was found in skin from newborn donors, whereas melanomas in adult skin were of a noninvasive in situ type only. This suggests that susceptibility of skin to exogenous tumor promoters is dependent on age. This is the first report on human cancer induction in vivo in which an imbalance of physiological factors combined with an environmental carcinogen can lead to transformation of normal tissue.

Introduction

The cause of melanoma, the deadliest form of skin cancer with a rising incidence worldwide, is unknown. Whereas UV radiation has been recognized to be a main risk factor for melanoma development, other factors have not been identified yet (1, 2). Epidemiological data show that sunburns in childhood impose a greater risk for melanoma development than sunburns in adulthood (3). It has been suggested that the proliferative activity of melanocytes that is higher in adolescent than in adult skin determines the transformation susceptibility to UV radiation. A synergistic activity of a melanocyte mitogen along with UVB for melanomagenesis has been demonstrated recently in a transgenic mouse model in which hepatocyte growth factor/scatter factor is overexpressed. In this model, neonates are significantly more susceptible to melanoma induction by UVB than adults (4). Hepatocyte growth factor is one of several known ligands for receptor tyrosine kinases that are expressed in the skin as environmental cues to support the survival and growth of melanocytes (5). In melanoma, constitutive activation of receptor tyrosine kinases plays a critical role in autonomous growth (6).

We have shown recently that the expression of another melanoma-associated receptor tyrosine kinase ligand, basic fibroblast growth factor (bFGF), along with UVB irradiation induced pigmented lesions in human skin (7). Increased protein expression of bFGF was achieved by injection of an adenoviral vector (Ad5) containing the bFGF gene into human skin grafted onto severe combined immunodeficiency disease mice. The increased production of bFGF by fibroblasts in the dermis stimulated the melanocytes in the epidermis. Whereas bFGF alone induced hyperpigmentation and proliferation of the melanocytes, only the combination of bFGF expression and UVB irradiation of the skin led to early stage transformation of melanocytes (7).

Here we tested whether a combined cutaneous expression of synergistic growth factors for melanocytes plays an etiologic role in melanomagenesis. We chose bFGF, the most important autocrine growth factor of melanoma, as well as stem cell factor (SCF) and endothelin-3 (ET-3). SCF and ET-3 are both essential for normal melanocyte development during embryogenesis, and support survival and growth of melanocytes synergistically (8–10). Along with the expression of the three growth factors, the human skin grafts were exposed to UVB irradiation at an erythemal dose three times weekly.

Materials and Methods

Cell Culture. Melanocytes, fibroblasts, and melanoma cells were isolated and cultured as described previously (7, 11). For anchorage-independent growth in soft agar, melanocytic cells were cultured in 0.35% Noble agar in melanocyte growth medium as described previously (12). Colonies were counted after 2 weeks. The expression of cadherins and melanoma-associated antigens was analyzed by fluorescence-activated flow cytometry using monoclonal antibodies against αvβ3 vitronectin receptor, Mel-CAM, melanotransferrin, chondroitin sulfate proteoglycan, and acetyl-GD3 (13).

Adenoviral Vectors for bFGF, Green Fluorescent Protein, LacZ, ET-3, and SCF. The adenoviral vector bFGF/Ad5 carrying the gene for the M, 18,000 form of the bFGF protein has been described (12). The control adenoviral vectors LacZ/Ad5 and green fluorescent protein/Ad5 (Vector Core, University of Pennsylvania, Philadelphia, PA) induce expression of the reporter gene β-galactosidase from Escherichia coli and green fluorescent protein, respectively. For construction of ET3/Ad5 and SCF/Ad5, total RNA from logarithmically growing human foreskin fibroblasts were isolated. Reverse transcription was performed with oligodeoxynucleotidic acid primer and SupperScript II RnaseH− Reverse Transcriptase (Life Technologies, Inc., Rockville, MD). Primers for PCR were designed specifically for SCF and ET-3 as follows: SCF (forward) 5'-GATCGACGGCCTGGTTTCTTCTATGAA-3'; SCF (reverse) 5'-TGCAACAGGGGTAAACATATAAATTGG-3'; ET-3 (forward) 5'-GCTCCGGCGCTGACCTGTCCCTTCTT-3'; ET-3 (reverse) 5'-CCTAAGGGGCTTCCTTCTTGAGAGG-3'. The amplified PCR fragments were cloned into pCR 2.1 (Invitrogen, Carlsbad, CA) and sequence verified using automated fluorescence sequencing (ABI, Foster City, CA). The cDNA fragments were then cloned into pShuttle-CMV, orientations of which were checked by restriction mapping and recombined with pADEasy-1 in E. coli strain B5183 essentially as described (14). The correct recombined clones were identified by restriction mapping, and the DNA was transfected into 293 cells. All of the vectors were serially amplified, purified through double cesium chloride centrifugation as described before (15), and titered to 1–5 × 109 plaque-forming units/ml.

Human skin grafts were injected intradermally with the adenoviral vectors at a concentration of 5 × 109 plaque-forming units in a total volume of 100 μl sterile PBS. Injections were performed once per week by the same person (R.T.).

Human Skin Grafting. Human foreskins from newborns, and abdominal, breast, and facial skin from adult donors who underwent plastic surgery...
(Cooperative Human Tissue Network, Philadelphia, PA) were prepared and grafted within 48 h of excision as described (7, 11). Grafts were well healed after 4–6 weeks and used for the experiments. The Wistar Institutional Animal Care and Use Committee approved all of the protocols.

UV Irradiation. UV light was provided by two Westinghouse FS72T12/UVB lamps (UV Resources International, Lakewood, OH) with a peak output at 313 nm and a range of 280–370 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 UVA. During irradiation, mice were separated from each other and allowed to move freely in the cage. Irradiation was performed three times weekly for ~10 min each time over a period of up to 4 weeks.

Histology, Immunohistochemistry, and Immunofluorescence. Preparation, fixation, and embedding of tissues were performed as described (7, 11). The DNA-binding fluorochrome Hoechst 33258 (Sigma, St. Louis, MO) was used to distinguish human from murine cells.

Immunohistochemistry and immunofluorescence techniques have been described previously (7, 11). Primary monoclonal and polyclonal antibodies used in this study were bFGF-8 (12), ET-3 (DPC Biermann, Bad Neuheim, Germany), FGFR1 (QED Bioscience Inc., San Diego, CA), SCF (IBL, Gunma, Japan), S100, CD117/c-kit (Dako, Carpinteria, CA), Ki-67 (clone MIB-1; Immunotech, Westbrook, ME), HMB45 (Biogenex, San Ramon, CA), Melan-A (clone A103; Novocastra, Newcastle upon Tyne, United Kingdom), NKIC3 (Monosan, Uden, the Netherlands), and Phospho-p44/42 mitogen-activated protein kinase (Thr202/Tyr204; Cell Signaling, Beverly, MA). A mouse IgG1 isotype antibody (P3) was used as negative control for each staining.

Mutation Analysis for N-RAS and BRAF. Serial sections were prepared as published previously (16). The sections were microdissected with a PixCell laser-capture microscope (Arcturus Engineering, Mountain View, CA), and DNA extracts were used for PCR. BRAF exon 15 was amplified by using primers as described previously (17) and for seminested PCR by using primer 5'-AATCATGGAATATAGCCTCA-3'. N-RAS exon 2 was amplified by using primers and conditions as described previously (16). Single-strand conformation polymorphism for BRAF analysis was done in the absence of glycerol at 5°C and 30 W. For each DNA extract, two or three independent PCR reactions were performed to confirm reproducibility. Each experiment was repeated three times. The sequences of the shifted bands were analyzed using outer primers in both directions as published previously (16).

Statistics. All of the data are expressed as mean ± SD of the mean of observations. Individual groups were compared with Student’s unpaired t test. P < 0.05 was considered significant.

Results and Discussion

Adenoviral vectors for SCF and ET-3 were constructed, and their transduction efficiency was tested in human cells in vitro (data not shown). SCF expression in human skin grafted to severe combined immunodeficiency disease mouse before (A) and 11 days after (B) beginning of SCF expression in the dermis. Pigmentation increased and black spots developed. C, hyperplasia of the epidermis and an increase in melanocytes (red cells) after four weekly injections of SCF/Ad5. D, no changes of the epidermis, normal number, and location of melanocytes (red cells) after four weekly injections of ET-3/Ad5. E, GFP is expressed beneath the epidermis (ep) after two weekly injections of GFP/Ad5. Sections are counterstained with the Hoechst fluorochrome that stains all nuclei blue. F, beginning nest formation (arrows) of melanocytes after four weekly injections of a combination of SCF/Ad5 and bFGF/Ad5. A and B, scale bar, 1 cm; C, D, and F, HMB45 staining; C–E, scale bar, 200 μm; F, scale bar, 100 μm.
significant melanocytic changes (Fig. 1D). The injected viral vectors spread throughout the dermis, never directly entering the epidermis (Fig. 1E) indicating that the growth factors affected the epidermal melanocytes in a paracrine manner as demonstrated for bFGF (7).

UVB irradiation of the human skin together with the cutaneous expression of ET-3, SCF, or bFGF had no additional effect on the melanocytes within a 4-week observation period. However, a combination of bFGF, ET-3, and SCF led to cluster formation of the melanocytes in the epidermo-dermal junction zone and to migration of single melanocytes into the upper layers of the epidermis within 2–4 weeks (Fig. 1F). Growth factors given individually could neither induce melanocytic nests nor migration of single melanocytes.

When the weekly induced expression of all three of the growth factors bFGF, ET-3, and SCF was combined with 30–50 mJ/cm² UVB irradiation three times weekly, severe pigmented lesions developed (Fig. 1E). Histologically these lesions were composed of cytologically atypical melanocytes and represented in situ melanomas (6 of 17; 35%) and invasive melanomas (11 of 17; 65%; Fig. 2, B–E) similar to those seen in patients. Of the 79 human skin grafts that received the combination of growth factors and UVB irradiation, pigmented lesions were clinically observed in 89%, and melanomas fitting the standard histopathologic criteria were observed in 34% (Table 1). Immunohistochemical analyses of the lesions revealed expression of the melanoma markers S100 (Fig. 2E), HMB45 (Fig. 2D), Melan-A, and NKIC3 (data not shown). They also expressed the growth factor receptors FGFR1 and c-kit (data not shown). Melanocytic cells isolated and cultured from these lesions formed colonies in soft agar (Fig. 2F) comparable with established melanoma cell lines (Fig. 2G), whereas normal human melanocytes isolated from human skin did not (data not shown). Flow cytometry analysis for melanoma cell surface markers associated with invasion and metastasis revealed expression of β3 integrin, melanoma cell adhesion molecule, melano-transferrin, chondroitin sulfate proteoglycan, and acetyl-GD3 in all of three cultures tested (data not shown). Whereas E-cadherin was also detected in these cultures, expression of N-cadherin was only found in melanocytic cell fractions isolated from the deeper parts of the skin, i.e., dermis and basement membrane, and in one of three melanocytic cell fractions isolated from the epidermis. This implies that a change from E- to N-cadherin expression occurred in the cells invading the skin, which is characteristic of invasive melanoma (18). These data provide new evidence that human melanoma can be induced by UVB, when melanocytes are activated through their growth factor receptors leading to uncontrolled growth and increased susceptibility to the carcinogenic effects of UV radiation. Because
melanoma lesions with nest formation, pagetoid growth, and invasion could not be induced by each growth factor alone, the data demonstrate that bFGF, ET-3, and SCF must have exerted synergistic effects on the melanocytes that cannot be explained by growth stimulation only. Possibly, induction of migration as shown for SCF in melanocytes in vitro was another pathogenic effect. Possible physiological causes for an increased receptor stimulation by activated growth factors include body surface expansion during adolescence or burns and wounds (19). Because the studies were done in skin specimens from newborns, we then investigated whether the observed effects on melanocytes were age-dependent. Adult skin grafts from the abdomen, breast, and face of 11 different donors were exposed to a combination of bFGF, ET-3, SCF, and UVB (n = 9); combination of bFGF, ET-3, and SCF without UVB (n = 11); UVB alone (n = 10); or were observed only (n = 8). After 4 weeks, in situ melanomas were found in 45–56% (n = 5, respectively) of the grafts exposed to the three growth factors independent from the exposure to UVB (Fig. 3, A and B), whereas there were no melanocytic lesions by UVB only or observation only. Compared with the results in newborn skin, melanomas in adult skin were less severe and noninvasive, but could be induced without UVB. These data indicates that young skin is more susceptible to the transforming effects of external growth factor receptor activation and UVB radiation, but that possibly pre-existing acquired mutations in adult skin predispose to melanoma development.

### Table 1 Pathologic changes in melanocytes in human skin grafts after 2–4 weeks exposure to different growth factor combinations and UVB

<table>
<thead>
<tr>
<th>Growth factors (n)</th>
<th>UVB</th>
<th>n</th>
<th>Pigmented lesion (%)</th>
<th>Increase of melanocytes</th>
<th>Suprabasal melanocytes</th>
<th>Melanocytic nests</th>
<th>Melanoma (%)</th>
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<tbody>
<tr>
<td><strong>bFGF + ET-3 + SCF</strong></td>
<td>3</td>
<td>7</td>
<td>70/79 (89)</td>
<td>4/5</td>
<td>21</td>
<td>5</td>
<td>17/50 (34)</td>
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<tr>
<td><strong>bFGF + ET-3</strong></td>
<td>2</td>
<td>7</td>
<td>6/7</td>
<td>4/5</td>
<td>8</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td><strong>bFGF + SCF</strong></td>
<td>2</td>
<td>7</td>
<td>3/5</td>
<td>2/5</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>ET-3 + SCF</strong></td>
<td>2</td>
<td>5</td>
<td>2/5</td>
<td>2/5</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>ET-3</strong></td>
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<td>5</td>
<td>0/5</td>
<td>0/5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>SCF</strong></td>
<td>1</td>
<td>5</td>
<td>0/5</td>
<td>0/5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>bFGF**</td>
<td>1</td>
<td>6</td>
<td>6/6</td>
<td>6/6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GFP/LacZ</td>
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<td>5</td>
<td>0/5</td>
<td>0/5</td>
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<td>0</td>
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</tbody>
</table>

* Growth factors basic fibroblast growth factor (bFGF), endothelin-3 (ET-3), and stem cell factor (SCF) as well as reporter genes green fluorescent protein (GFP) or LacZ were expressed in human skin grafts by weekly injections of the respective adenoaviral vectors. The total dose of each injection was 5 × 10⁶ plaque forming units per graft in each group regardless of the number of growth factors used.

* Detection of at least one brown or black spot on the skin within 2–4 weeks of treatment.

* Significant increase in HMB45-positive cells compared with non-treated skin grafts.

* Average number of melanocytic cells per histological section that have left the basement membrane and migrated into the epidermis.

* Average number of melanocytic nests per histological section. One nest is defined as a cluster of at least 4 melanocytic cells.

* Histopathological criteria are fulfilled to classify lesion as melanoma or melanoma in situ.

* Data published (7). One melanoma was found after 2.5 months.

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**Fig. 3.** In situ melanomas (arrows) in human adult skin grafts 4 weeks after exposure to basic fibroblast growth factor (bFGF), endothelin-3 (ET-3), and stem cell factor (SCF) only (A) and along with UVB irradiation (B). H&E staining, scale bar, 100 μm. Activation of RAS-RAF-MAPK pathway. C, Immunohistochemical detection of phosphorylation of extracellular signal-regulated kinase 1 (red) in a melanoma lesion induced by bFGF, ET-3, SCF, and UVB in a foreskin graft, using Phospho-p44/42 mitogen-activated protein kinase antibody. Scale bar, 50 μm. D, Single-strand conformation polymorphism analysis of PCR products derived from exon 15 of BRAF. Lanes 1 and 2, controls for BRAF codon 599. Melanoma cell line 224 (Lane 1) is wild-type (Wt) with the sequence GTG (VAL), and melanoma cell line A375 (Lane 2) is hemizygous for a GAG (Glu) substitution. Lanes 3 and 4, DNA extract from two melanocytic skin lesions induced by bFGF, ET-3, and SCF only (Lane 3) or by bFGF, ET-3, SCF, and UVB (Lane 4) showing a bandshift with a GAG (Glu) mutation in codon 599.
triggered by receptor tyrosine kinase activation only. The detection of phosphorylation of extracellular signal-regulated kinase 1 in all of the analyzed invasive melanoma lesions (5 of 5) by immunohistochemistry (Fig. 3C) indicates that the RAS-RAF-extracellular signal-regulated kinase-mitogen-activated protein kinase pathway was activated in the transformed cells, mostly likely through the external growth factor stimulation. Activating mutations of the N-RAS proto-oncogene and, more recently, of the BRAF gene leading to constitutive activation of the RAS-RAF-extracellular signal-regulated kinase-mitogen-activated protein kinase pathway have been described in human melanoma (16, 17). We analyzed the adult skin and newborn skin lesions for these mutations. There were no mutations in N-RAS codon 61 in melanoma cells of microdissected adult skin nor in newborn skin lesions. BRAF was not mutated in 10 of 10 analyzed adult skin lesions except for a silence mutation in 1 sample (TCTG01TCC). However, in 2 of 5 analyzed newborn skin lesions, an activating V599E BRAF mutation in exon 15 was found in a small proportion of the cells (Fig. 3D). Because the BRAF mutation does not display a UV signature mutation site, it may have pre-existed or it may have been induced by yet unidentified mechanisms. Genetic alterations induced by UVB may only occur at time points later than 4 weeks (20).

Although melanomas could be induced in our model fitting standard histopathologic criteria, long-term observation of the skin grafts showed that the lesions regressed upon withdrawal of the growth factor stimulation after 4 weeks. Similarly, the colony formation in soft agar of the melanocytic cells isolated from the lesions was dependent on exogenous growth factor supplementation, and the life span of these cells in culture was limited, albeit extended. These observations indicate that the lesions have not acquired enough stable mutations or telomere stabilization yet that renders them independent of growth factor stimulation. Activating mutations of the N-RAS proto-oncogene and, more recently, of the BRAF gene leading to constitutive activation of the RAS-RAF-extracellular signal-regulated kinase-mitogen-activated protein kinase pathway was activated in the transformed cells, mostly likely through the external growth factor stimulation. Activating mutations of the N-RAS proto-oncogene and, more recently, of the BRAF gene leading to constitutive activation of the RAS-RAF-extracellular signal-regulated kinase-mitogen-activated protein kinase pathway have been described in human melanoma (16, 17). We analyzed the adult skin and newborn skin lesions for these mutations. There were no mutations in N-RAS codon 61 in melanoma cells of microdissected adult skin nor in newborn skin lesions. BRAF was not mutated in 10 of 10 analyzed adult skin lesions except for a silence mutation in 1 sample (TCTG01TCC). However, in 2 of 5 analyzed newborn skin lesions, an activating V599E BRAF mutation in exon 15 was found in a small proportion of the cells (Fig. 3D). Because the BRAF mutation does not display a UV signature mutation site, it may have pre-existed or it may have been induced by yet unidentified mechanisms. Genetic alterations induced by UVB may only occur at time points later than 4 weeks (20).

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We conclude that a melanoma phenotype can be induced in human skin by a combination of three growth factors and UVB within a very short period of time, but additional factors or an extended time of growth stimulation and UVB irradiation are needed for the acquisition of stable genetic alterations that are required for autonomous growth and unlimited life span.

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References

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