Molecular Analysis of Melanoma Precursor Lesions

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Abstract

Atypical (dysplastic) nevi are melanocytic lesions, which are precursors of melanoma as well as markers of increased melanoma risk. Although these lesions exhibit distinct clinical and histological features, their molecular features are largely unknown. To determine whether atypical, compared to benign nevi, from patients with a clinical history of malignant melanoma reveal molecular changes, we analyzed these lesions for the expression of two growth factors (basic fibroblast growth factor and transforming growth factor α), their receptors (fibroblast growth factor receptor-1 and epidermal growth factor receptor), and two cell adhesion molecules (MUC18 and αvβ3 integrin), all of which are expressed in primary and metastatic melanomas. The results demonstrated a statistically significant correlation (P = 0.02) between increasing degrees of histological atypia and expression of epidermal growth factor receptor in the epidermal keratinocytes of atypical melanocytic lesions. Furthermore, both atypical and benign nevi revealed considerably high levels of overall gene activity in their dermal melanocytic and epidermal keratinocytic compartments. In contrast, the epidermal-dermal junction wherein melanoma evolves showed little gene activity, suggesting that molecular events occurring adjacent to this junction may be important for melanocytic transformation.

Introduction

Human melanoma represents the end point in a series of progressive stages of melanocyte transformation. In a significant number of patients, this transformation process begins with melanocytes of cutaneous nevi designated as atypical (dysplastic) nevi, which can progress to primary melanoma in the radial growth phase to primary melanoma in the vertical growth phase to metastatic melanoma (Refs. 1–3; reviewed in Ref. 4). In the setting of familial melanoma, comprising about 10% of the overall incidence of melanoma, the presence of atypical nevi is associated with a nearly 100% risk of developing primary melanoma by age 70 (5, 6). However, atypical nevi also occur outside the familial melanoma setting (7, 8), and it is currently estimated that 40–60% of sporadic melanomas develop from these melanocytic precursor lesions. For example, patients with a clinical history of primary melanoma and two or more atypical nevi are at an 8-fold greater risk for developing a second primary melanoma (9).

The criteria for identifying and distinguishing atypical from benign nevi are based upon a combination of clinical and histopathological features (Ref. 10; reviewed in Ref. 11). The most apparent clinical features of atypical nevi are manifest in their size (≥5 mm in diameter), irregular borders, and variegated pigmentation. Their prevalent architectural features are basalar melanocytic hyperplasia and bridging and confluence of the rete ridges. Furthermore, atypical nevi exhibit cytological features of nuclear pleomorphism and hyperchromatism of nuclei and lymphocytic infiltrate of the dermis (11). In contrast, benign nevi are generally less than 4 mm in diameter, displaying regular and defined borders and uniform coloration. Moreover, benign melanocytic lesions do not exhibit architectural and cytological features of atypia.

The clinical prognosis for patients with melanoma is directly related to the depth of invasion of the primary lesion at the time of diagnosis. Thus, when recognized early in the biological course of the disease, the patient is often cured by a wide and deep excision of the melanoma. However, once a primary melanoma in the vertical growth phase metastasizes to regional lymph nodes or distant sites, the prognosis is grave because conventional chemotherapy or radiation treatment do not reliably affect the course of the disease. For this reason, it is of great importance to gain an insight into molecular events that govern the early stages of melanocytic atypia, thereby making it possible to implement strategies that will interfere with progression to advanced-stage melanomas.

To date, little information is available regarding genes that are activated and expressed in atypical nevi. In contrast, over the past decade, significant information has accumulated regarding molecular features of primary and metastatic melanomas. In particular, these investigations have focused upon genes required for the proliferation and cell adhesion properties of malignant melanomas (reviewed in Ref. 12). For
example, bFGF³ and one of its receptors, FGFR-1, are expressed in all primary and metastatic melanomas analyzed to date. Their expression is essential for the proliferation of malignant melanomas, both in vitro and in vivo (13-15). On the other hand, TGF-α and its receptor, EGFR, are expressed in some but not all melanomas. However, previous studies suggested that the expression of TGF-α and EGFR increases with progression to advanced-stage melanoma (16, 17). Likewise, MUC18, a member of the cell adhesion molecules of the immunoglobulin superfamily (18), and the αvβ3 subunit of integrin (19) were reported previously to represent progression markers of primary and metastatic melanomas (18, 20-22).

To determine whether activation and expression of any of these six genes would correlate with the onset of melanocytic progression in atypical nevi, we obtained atypical nevi and, as a control, benign nevi from patients with a clinical history of melanoma. By analyzing the epidermal, junctional, dermal, stromal, and vascular compartments of these nevi for the expression of each of these genes, we observed a statistically significant correlation between increasing degrees of histopathological atypia and expression of EGFR in the EKTs of atypical nevi. Furthermore, atypical as well as benign nevi revealed high levels of gene activity in their EKts and DNVs, which are clusters of melanocytes. In contrast, overall gene activity in the STR, VAS, and specifically, the JCT of these melanocytic lesions was low. The latter observation is particularly surprising because melanoma is thought to evolve in the junction.

Results
Clinical and Histopathological Assessment of Atypical and Benign Nevi. As listed in Table 1, the established clinical features of atypical nevi are: size ≥5 mm in diameter, asymmetry, irregular borders, variegated pigmentation, and the presence of a macular surface component (11). In contrast, benign nevi are typically smaller than 5 mm in diameter, have regular contours and smooth borders, and exhibit uniform pigmentation. In concordance with these features, we obtained a total of 78 nevi from 30 patients with a clinical history of melanoma who had not undergone systemic treatment for their disease prior to the removal of nevi. In the case of each patient, we removed at least one nevus that exhibited the most pronounced features of clinical atypia and one nevus that showed no clinical signs of atypia. Upon excision, each nevus was divided, with one portion set aside for histopathological analysis, while the other was snap-frozen for subsequent molecular analyses. The degree of architectural and cytological atypia was determined for each nevus spec-

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³ The abbreviations used are: bFGF, basic fibroblast growth factor; FGFR, fibroblast growth factor receptor; TGF, transforming growth factor; EGFR, epidermal growth factor receptor; EKT, epidermal keratinocyte; JCT, epidermal-dermal junction; DNV, dermal nevocyte; STR, stroma; VAS, vasculature.

⁴ Y. Wang and D. Becker, Antisense targeting of bFGF/FGFR-1 in human melanomas inhibits intratumoral angiogenesis and tumor growth, submitted for publication.

Table 1 Clinical and histopathological features of atypical nevi

Expression of Growth Factors/Growth Factor Receptors and Cell Adhesion Molecules in Atypical and Benign Nevi. To determine whether atypical compared to benign melanocytic lesions express genes demonstrated previously or suggested to play an important role in the proliferation and cell adhesion of advanced-stage melanomas, we used antibodies specific for each of these proteins. To detect expression of bFGF and FGFR-1, we carried out in situ hybridization with human bFGF and FGFR-1-specific riboprobes because the currently available antibodies for bFGF and FGFR-1 do not work well in immunohistochemistry. Moreover, the available antibodies do not distinguish between FGFR-1 and the other three members of the FGFR family, e.g., FGFR-2, FGFR-3, and FGFR-4.

An example of the results of these in situ hybridization and immunohistochemical analyses in an atypical nevus is depicted in Fig. 1. This particular nevus (Fig. 1A), histopathologically determined to exhibit signs of an intermediate degree of architectural and cytological atypia (grade 2; Fig. 1, B and C), revealed strong expression of bFGF (Fig. 1E) and FGFR-1-specific (Fig. 1G) mRNA in its DNVs and in the STR. In contrast, TGF-α was not expressed in the DNVs (Fig. 1H), whereas EGFR was strongly expressed in the EKts (Fig. 1f). Like TGF-α, the cell adhesion molecules MUC18 (Fig. 1J) and αvβ3 integrin (Fig. 1K) were not detected in the DNVs of this atypical melanocytic lesion. As a control, we used an isotype-specific (mouse IgG1) antibody, which did not stain the

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2 The abbreviations used are: bFGF, basic fibroblast growth factor; FGFR, fibroblast growth factor receptor; TGF, transforming growth factor; EGFR, epidermal growth factor receptor; EKT, epidermal keratinocyte; JCT, epidermal-dermal junction; DNV, dermal nevocyte; STR, stroma; VAS, vasculature.

cells of this or any of the other nevi evaluated (data not shown).

Fig. 2 documents the presence or absence of each of the six molecules in the EKTs, the JCT, the DNVs, the STR, and the VAS of all nevi analyzed in this study. As shown in Fig. 2A, bFGF and FGFR-1 were predominantly expressed in the DNVs and STR of atypical and benign nevi, whereas they revealed little or no expression in the epidermis, junction, or VAS. On the other hand, expression of TGF-α (Fig. 2B) was predominantly detected in EKTs, in the junction, and in DNVs. Most surprising were the results obtained for EGFR (Fig. 2B). This gene was almost exclusively expressed in the epidermis of all nevi. Regarding the presence of the two cell adhesion molecules, MUC18 and αvβ3 integrin, the former was predominantly detected in DNVs, with little expression noted in EKTs and in the junction (Fig. 2C). In contrast, αvβ3 integrin was found to be mostly expressed in blood vessels, specifically in the case of nevi with an intermediate and high degree of atypia (grades 2 and 3; Fig. 2C). Interestingly, neither adhesion molecule was present in the STR, which is mostly comprised of fibroblasts.

Upon establishing the presence or absence of each molecule in the five different tissue compartments of each nevus
Fig. 2. Percentage of benign nevi and nevi with increasing degrees of histological atypia showing expression of bFGF, FGFR-1, TGF-α, EGFR, MUC18, and αvβ3 integrin. A, percentage of nevi with no signs of histological atypia (grade 0), a low degree of histological atypia (grade 1), an intermediate degree of histological atypia (grade 2), and a high degree of histological atypia (grade 3), which revealed expression of bFGF and FGFR-1 in their EKTs, JCT, DNVs, STR, and their VAS. B and C, a list in a similar fashion of the different groups of nevi that demonstrated expression of TGF-α (B), EGFR (B), MUC18 (C), and αvβ3 integrin (C).
Fig. 3. Percentage of gene expression in the individual tissue compartments of atypical compared to benign nevi. The upper panel documents the percentage of bFGF, FGFR-1, TGF-α, EGFR, MUC18, and αvβ3 integrin expression in the EKTs, JCT, DNVs, STR, and VAS of the combined group of atypical nevi with an intermediate (grade 2) and high degree (grade 3) of histological atypia. Listed in the panel below is the expression of each molecule in the five different tissue compartments of the combined group of benign melanocytic lesions with no signs of histological atypia (grade 0) and a low degree (grade 1) of histological atypia.

Specimen, we applied the Fisher Exact Test to determine whether expression of any of these genes correlated with increasing degrees of histopathological atypia. Because nevi with no signs of histopathological atypia (grade 0) and nevi with a low degree of histopathological atypia (grade 1) do not significantly differ from each other in their histological features, we combined, for the purpose of statistical analysis, the total number of nevi from these two categories and compared them to the total number of nevi with an intermediate (grade 2) and high degree (grade 3) of histopathological atypia. The results of the Fisher Exact Test revealed a significant correlation (P = 0.02) between expression of EGFR in EKTs of atypical but not benign melanocytic lesions.

Summarized in Fig. 3 are the results of the statistical analyses, which list by tissue compartment the percentage of gene expression in atypical nevi versus the percentage of gene expression in benign nevi. In addition to revealing a correlation between epidermal expression of EGFR and increasing signs of histological atypia, the data pointed to two other interesting observations: (a) although we did not find a statistically relevant correlation between expression of the other five genes and increasing degrees of histological atypia, expression of MUC18 in DNVs and expression of αvβ3 integrin in the VAS of both atypical and benign nevi were more prominent than expression of bFGF or FGFR-1; and (b) the percentage of overall gene activity in all nevi was highest in the epidermis and in the DNVs, whereas the JCT revealed the lowest level of overall gene activity. This finding was rather unexpected, particularly in the case of atypical nevi, for the reason that melanoma is thought to arise in the junctional zone. Thus, one may have predicted that this tissue compartment, which is mostly
comprised of melanocytes, would show a higher or the highest level of overall gene activity, i.e., gene expression.

Discussion

The data presented in this study provide a first insight into the status of melanoma-associated biological markers in atypical versus benign melanocytic lesions. Having analyzed a total of 78 nevi from 30 patients with a clinical history of melanoma for the expression of two different growth factors, their receptors, and two different cell adhesion molecules, the following findings were obtained. With respect to the growth factors, bFGF and TGF-α, and their corresponding receptors, FGFR-1 and EGFR, only EGFR was found to be expressed at a significantly higher percentage (P = 0.02) in the epidermis of atypical versus benign nevi, suggesting that expression of this gene may correlate with and thus serve as a marker of early melanocytic progression. The observation that EGFR was strongly expressed in the EKTs of every atypical nevus evaluated in this study is quite surprising, given the fact that less than 10% of primary and metastatic melanoma cell lines express this gene, although it was suggested previously that EGFR is a putative progression marker of malignant melanomas (17). Moreover, inhibition of expression of EGFR in EGFR-positive melanoma cell lines does not impair their proliferation. To explain this somewhat dichotomous pattern of EGFR expression in atypical nevi versus malignant melanomas, one might postulate that EGFR-mediated signal transduction in EKTs of melanoma precursor lesions reflects the action of a paracrine pathway required for the proliferation of epidermal and/or junctional melanocytes, and that this pathway is no longer needed to sustain the growth of these cells once they have progressed to malignant melanoma. In contrast, both bFGF and FGFR-1 are expressed at high levels in all primary and metastatic melanoma cell lines and specimens analyzed to date (15, 23), and they are essential mediators of the proliferation of malignant melanomas (13–15, 24). On the other hand, the results of the present study did not provide evidence for a correlation between increasing degrees of histopathological atypia and increasing expression of bFGF or FGFR-1 in any of the five tissue compartments of atypical nevi. Thus, one may hypothesize that this particular growth factor ligand-receptor pair is not required for the proliferation of melanocytes in their early stages of tumor progression but only for their progression to and maintenance of the more advanced stages of melanoma. It is also possible that expression of bFGF/FGFR-1 is not a requisite for the onset of malignant transformation in the human melanocytic system but instead, as suggested previously for bFGF (25), is related to the process of tumor invasion.

Although atypical compared to benign nevi did not demonstrate a higher percentage of MUC18 or αβ3 integrin expression, it is interesting to note that the cell-cell adhesion molecule MUC18 was predominantly detected in the DNVs of all lesions, whereas the cell-matrix adhesion molecule, αβ3 integrin, was almost exclusively expressed in their vessels. With respect to the expression of MUC18 in the different progression stages of the human melanocytic system, previous studies suggested that this gene is only sporadically expressed in benign nevi and melanomas in the radial growth phase, whereas it was detected in 80% of primary melanomas in the vertical growth phase and in metastatic melanomas (18). Furthermore, it was concluded that high-level expression of MUC18 in primary melanomas in the vertical growth phase correlates with poor prognosis and the competence for metastasis. Although we have not yet analyzed tissue sections representing radial growth phase melanomas, the data of our study demonstrated a relatively high percentage of atypical and benign melanocytic lesions to express MUC18 in their DNVs.

Like MUC18, expression of the β3 subunit of integrin was documented previously to be restricted to melanomas in the vertical and metastatic growth phase, whereas the αv subunit was found to be expressed in benign nevi and melanomas (21). Concordant with the observation that expression of αβ3 integrin is preferentially detected in angiogenic blood vessels (26), our findings demonstrated strong expression of αβ3 integrin in the VAS of both atypical and benign lesions. Interestingly, it was recently documented that when cultured on a dermal collagen matrix, melanoma cells that failed to express αβ3 integrin underwent apoptosis (27), suggesting that αβ3 integrin expression in advanced-stage melanomas may promote their survival by anchoring the cells to the extracellular matrix.

Finally, another interesting observation that emerged from these studies was the finding that the levels of overall gene activity in atypical as well as benign nevi were highest in their EKTs and DNVs. In contrast, both categories of melanocytic lesions revealed the lowest level of overall gene activity in their JCT (Fig. 3). Particularly in the case of atypical nevi, the latter result was quite unexpected because the junction, which is composed of melanocytes, represents the tissue compartment wherein melanoma is thought to evolve (4). Thus, one may have anticipated to find a higher level of overall gene activity in this junctional area. Instead, we found that the epidermis above and the DNVs below the junction emerged as the regions with the highest percentage of gene expression. In view of this finding, one could propose the existence of a paracrine mechanism whereby the gene products, present in the epidermis and DNVs, act as a support system for the melanocytes residing in the junction. Although not mutually exclusive, it is also possible that genes and gene products, other than the ones investigated, are prominently expressed in the junctional melanocytes of atypical nevi, and that it is these hitherto unknown genes that govern the progression to radial and vertical growth phase melanomas.

Materials and Methods

Collection and Preparation of Nevus Specimens. Benign nevi and nevi with varied clinical features of atypia were obtained from patients with a clinical history of melanoma (University of Pittsburgh Cancer Institute protocols 91-10 and 94-33; Institutional Review Board protocols 950642 and 950487), who were seen in the Melanoma Center of the University of Pittsburgh Cancer Institute. All nevi were photographed prior to their

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5 U. Rodeck, personal communication.
surgical removal either as punch biopsies or excisional biopsies. Nevi were only obtained from melanoma patients who had not undergone systemic treatment for their disease prior to the removal of nevi. Shortly after excision, each nevus specimen was divided; one part was immediately snap-frozen, while the other part was fixed in formalin and embedded in paraffin. H&E-stained tissue sections, prepared from two adjacent center sections, one snap-frozen, the other formalin-fixed and paraffin-embedded, were used to determine the degree of architectural and cytological atypia of each nevus.

**In Situ Hybridization.** A 520-bp and, likewise, a 100-bp bFGF-specific cDNA fragment, spanning nucleotides 1–520 and 1210–1400, respectively, of the human bFGF gene (28) were subcloned into the plasmid pBluescript. Similarly, a 140-bp FGFR-1-specific cDNA fragment, spanning the transmembrane region (nucleotides 1050–1190) of the human FGFR-1 gene (29) was subcloned into pBluescript. Antisense and control sense-oriented riboprobes were generated by in vitro transcription in the presence of digoxigenin-UTP (Boehringer Mannheim). Frozen sections (5 μm), embedded in OCT, were fixed in 4% paraformaldehyde, rehydrated, acetylated, and pretreated as described (30). Prehybridizations and hybridizations to digoxigenin-UTP-labeled riboprobes were performed at 50°C in buffer containing 50% formamide, 2× SSC, 20 mM Tris, 1× Denhardt’s solution, 1× EDTA, 10% dextran sulfate, and 500 μg/ml yeast RNA. Following hybridization, the specimens were washed at 42°C with 2× SSC for 30 min, 0.5× SSC for 15 min, and with 0.1× SSC for 30 min. Thereupon, the tissue sections were processed for immunological detection with anti-digoxigenin alkaline phosphatase conjugate and nitro blue tetrazolium substrate (Boehringer Mannheim), giving rise to a blue hybridization signal.

**Immunohistochemistry.** Acetone-fixed 5-μm tissue sections were rehydrated in PBS for 15 min. Upon preincubation in the presence of horse serum, the sections were incubated for 2 h with the respective primary antibodies. Thereafter, the tissue sections were rinsed with PBS and incubated for 30 min with biotinylated horse antimouse IgG secondary antibody, followed by rinses with PBS and a 30-min incubation in the presence of a peroxidase-conjugated avidin-biotin complex (Vector Laboratories). Red color was developed with amine ethol carbazol (Sigma Chemical Co.). The primary antibodies used in these studies were: mouse antihuman TGF-α monoclonal antibody (Oncogene Sciences), mouse antihuman EGFR monoclonal antibody (31), mouse antihuman MUC18 monoclonal antibody (32), and mouse antihuman αvβ6 integrin monoclonal antibody (33). In the case of each nevus specimen, a mouse IgG1 antibody (Tago) was used as an isotype control.

**Evaluation of Histopathological and Molecular Features.** Based upon established histopathological criteria (Table 1; Ref. 11), a grading scheme on a scale from 0–3 was used to determine the degree of architectural and cytological atypia of each nevus specimen. Nevi were scored positive for the presence of a hybridization signal in their individual tissue compartments when a signal was clearly visible and negative in the absence of a hybridization signal. All evaluations were performed by the same group of five individuals.

**Statistical Analysis.** For statistical evaluation of the data obtained in this study, the number of nevi with no signs of histological atypia (grade 0; 24 nevi) and those with a low degree of histological atypia (grade 1; 43 nevi) were combined in one group. The nevi in this group were compared to a second group, encompassing nevi with an intermediate (grade 2; 8 nevi) and a high degree of histological atypia (grade 3; 3 nevi). The results of the statistical analyses were based on the Fisher Exact Test.

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**References**


