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Original Articles

Macroscopic Spectral Imaging and Gene Expression Analysis of the Early Stages of Melanoma

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Abstract

Background: The stages of melanocytic progression are defined as atypical (dysplastic) nevus, melanoma in situ, melanoma in the radial growth phase (RGP), melanoma in the vertical growth phase (VGP), and melanoma in the metastatic growth phase (MGP). Melanoma in situ and RGP melanoma often develop in contiguous association with atypical nevi. This frequently poses a problem with respect to their early detection. Furthermore, unlike cells obtained from VGP and MGP melanomas, cells derived from melanoma in situ and RGP melanoma do not proliferate in vitro. Thus, compared to the late stages of the disease, less information is available regarding genes expressed in the early stages.

Materials and Methods: To determine whether spectral imaging, a recently developed optical imaging technique, can detect melanoma in situ and RGP melanoma arising in melanoma precursor lesions, atypical nevi in patients with a clinical history of melanoma were subjected to noninvasive macroscopic spectral imaging. To determine at what stage in the progression pathway of melanoma genes having important biological functions in VGP and MGP melanomas are activated and expressed, lesions of melanoma in situ were analyzed by immunohistochemistry and in situ hybridization for expression of some of these known molecular and immunologic markers.

Results: The present study demonstrates the capability of noninvasive spectral imaging to detect melanoma in situ and RGP melanoma that arise in contiguous association with atypical nevi. Furthermore, the study provides evidence that genes and antigens expressed in VGP and MGP melanoma are also expressed in melanoma in situ.

Conclusions: Because of the dark and variegated pigmentation of atypical nevi, melanoma in situ and RGP melanoma that arise in these melanoma precursor lesions are often difficult to recognize and thus frequently go unnoticed. The application of new optical screening techniques for early detection of melanoma and the identification of genes expressed in the early stages of melanoma development are two important avenues in the pursuit of melanoma prevention. The investigations presented here document that macroscopic spectral imaging has the potential to detect melanoma in its early stage of development and that genes essential for the proliferation and cell adhesion of VGP and MGP melanoma are already expressed in melanoma in situ.

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Introduction

Human melanoma exhibits three important features: first, it occurs sporadically in approximately 90% of cases, and is the result of familial

predisposition in about 10% of cases; second, both the sporadic and familial forms of melanoma in >60% of all cases evolve in a stepwise fashion; and third, the prognosis for patients with melanoma is directly related to the depth of invasion of the primary lesion at the time of diagnosis. Thus, when diagnosed at an early stage, the patient is often cured by a wide and deep excision of the melanoma. However, once vertical growth phase (VGP) melanoma metastasizes to regional lymph nodes or distant sites, the disease becomes refractory to conventional treatment. For this reason, it is important to explore and implement new noninvasive optical imaging techniques that can detect melanoma in the early stages of development.

Recent advances in the field of optical imaging have led to the development of new noninvasive imaging techniques that are expected to have a major impact on biomedical diagnostics and, thus, disease prevention. One of these recently developed optical imaging techniques is spectral imaging, which is defined as the application of spatially resolved spectroscopic analyses to macroscopic and microscopic samples (1,2). To determine whether noninvasive spectral imaging can detect melanoma in situ and radial growth phase (RGP) melanoma that arise in, or in contiguous association with, atypical nevi, we imaged atypical nevi in patients with a clinical history of melanoma. The data presented here provide examples of the capability of macroscopic spectral imaging to detect melanoma in atypical nevocytic lesions.

To gain an understanding of molecular events that govern the onset and progression of melanoma, two major research avenues have been pursued to date. First, genes have been identified whose expression is altered in VGP and metastatic growth phase (MGP) melanomas compared to normal melanocytes. Second, cytogenetic aberrations, detected in a large number of VGP and MGP melanomas of both familial and sporadic origin, have guided the identification of chromosomal loci harboring genes that are expressed in normal melanocytes but are deleted or inactivated in advanced-stage melanomas.

Most investigations pertaining to the study of gene expression and gene regulation in VGP and MGP melanomas have been conducted with established cell lines, and in the case of normal human melanocytes, with primary cell cultures. Availability of these cell lines and primary cell cultures has made it possible to study changes in gene expression that occur concomitantly with progression to the advanced stages of melanoma. For example, in order to proliferate in vitro, normal melanocytes require the presence of exogenous basic fibroblast growth factor (bFGF). In contrast, VGP and MGP melanomas produce their own bFGF (3). Cell–cell and cell–matrix adhesion molecules, such as cadherins and integrins, represent another example of genes that have been shown to be differentially expressed in normal melanocytes in contrast to VGP and MGP melanomas (3).

The knowledge of molecular and immunologic markers that are differentially expressed in normal melanocytes compared to advanced-stage melanomas raises the following question: at what stage in the melanocytic progression pathway do these changes occur? Since cells representing melanoma in situ and RGP melanoma do not proliferate in vitro, one can only obtain an answer for this question by analyzing specimens representing these early stages of melanoma development. Using probes for genes and antigens, that are expressed and present in advanced-stage melanomas, we performed immunohistochemical and in situ hybridization analysis of melanomas in situ, including melanomas in situ that developed in association with atypical nevi. The expression profile of a growth factor–growth factor receptor pair, cell adhesion molecules, and vascular, T cell, and dendritic cell markers detected in these melanomas in situ was similar to the expression status of these genes and antigens in VGP and MGP melanomas.

Materials and Methods

Macroscopic Spectral Imaging Analysis

Macroscopic spectral images of melanocytic lesions were acquired in vivo with a spectral imaging lens having a focal distance of about 20 cm that was attached to spectral imaging units previously described (4–6). The spectral images, forming a cube corresponding to 12–40 wavelength bands (between 440 and 700 nm), were transferred to a computer where they were converted from transmission to optical density. A linear unmixing algorithm, implemented in custom-designed spectral imaging analysis software [Applied Spectral Imaging (ASI), Migdal HaEmek, Israel; (2)], was applied to each image, and segmented regions of the image sets were pseudocolored for optimal display contrast.

Collection and Processing of Biopsy Specimens

In compliance with approved clinical protocols [University of Pittsburgh Cancer Institute (UPCI) protocols 91–10 and 96–39; Institutional Review Board (IRB) protocols 950642 and 950497], snap-frozen, and formalin-fixed/paraffin-embedded specimens representing normal skin, benign nevi, atypical nevi, and melanomas were obtained from patients who were seen at the Melanoma Center of the University of Pittsburgh Cancer Institute and from the Department of Pathology at the University of Pittsburgh Medical Center (UPMC). The absence or presence and degree of architectural and cytologic atypia of each specimen were determined according to established histopathologic criteria (7). All specimens were obtained from melanoma patients prior to any treatment for their disease.

Immunohistochemistry and In Situ Hybridization

Snap-frozen, and formalin-fixed/paraffin-embedded 5-μM tissue sections were analyzed by immunohistochemistry as previously described (8–10). A monoclonal antibody to human fibroblast growth factor receptor 1 (FGFR-1) was obtained from Dr. D. Larocca (SelectiveGenetics, San Diego, CA), and monoclonal antibodies to MAGE-1 and CD83 were provided by Dr. W. Storkus (University of Pittsburgh, Pittsburgh, PA). A polyclonal antibody to E-cadherin was obtained from Dr. M. Herlyn (The Wistar Institute, Philadelphia, PA). Polyclonal antibodies to the following proteins and antigens were purchased from commercial suppliers: bFGF was from BioGenex (San Ramon, CA), N-cadherin was from Sigma (St. Louis, MO), and CD3, CD4, CD8, CD31, and S100 were from Dako Corporation (Carpinteria, CA).

Using a biotinylated 470 base pair (bp) human bFGF cDNA fragment and a 140 bp biotinylated human FGFR-1 cDNA probe, in situ hybridizations of snap-frozen, and formalin-fixed/paraffin-embedded 5-μM normal skin, nevus, and melanoma sections were performed as previously described (8,9).

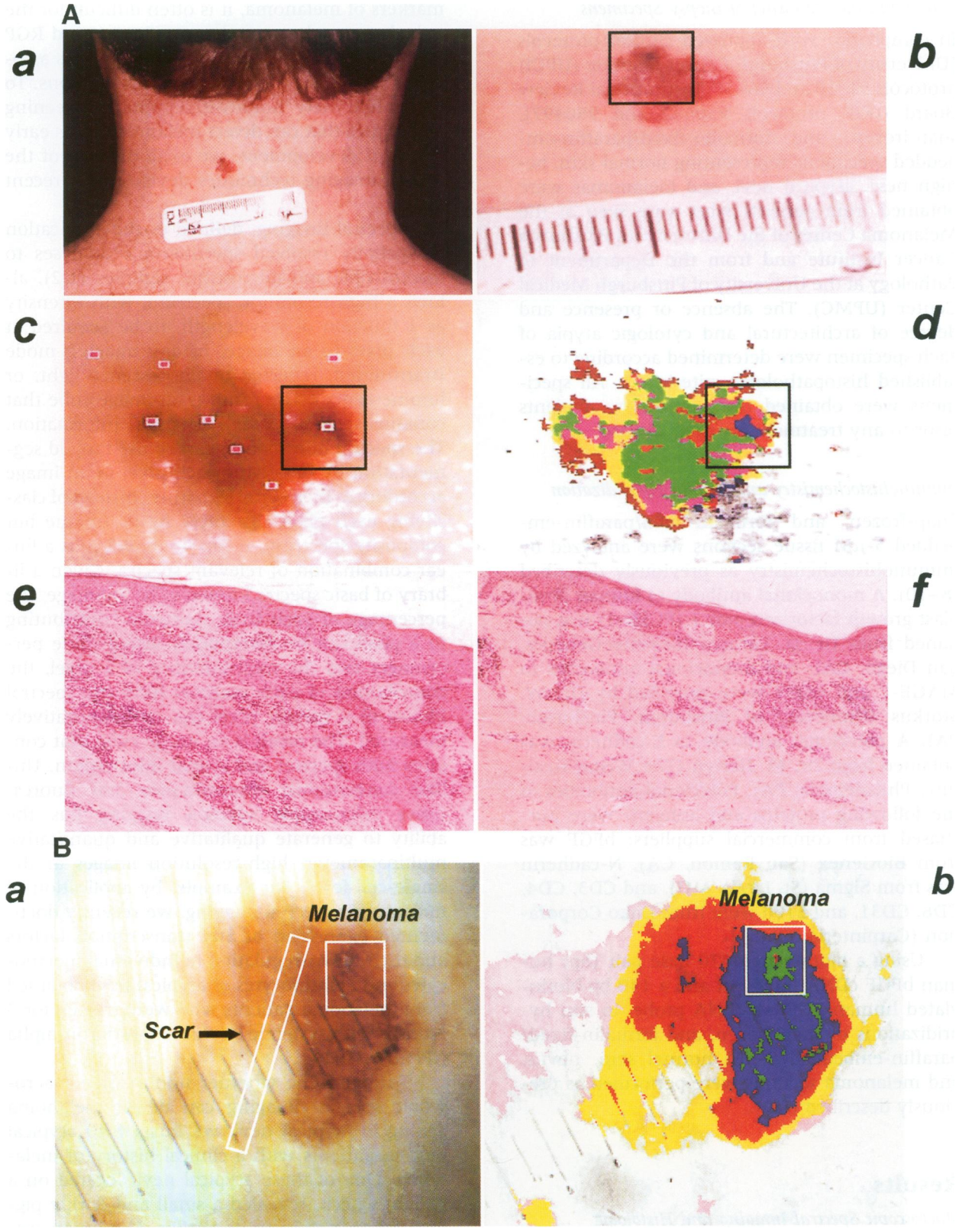
Results

Macroscopic Spectral Imaging and Histologic Analysis of Melanoma In Situ and RGP Melanoma Arising in Atypical Nevi

Given the dark and variegated pigmentation of atypical nevi, which are the precursors and risk markers of melanoma, it is often difficult for the naked eye to detect melanoma in situ and RGP melanoma developing in, or in contiguous association with, these atypical nevocytic lesions. To implement new noninvasive optical screening techniques that can detect melanoma in its early stages of development, we explored one of the optical imaging techniques developed in recent years.

Spectral imaging, defined as the application of spatially resolved spectroscopic analyses to macroscopic and microscopic samples (1,2), allows a high-resolution spectrum, with intensity as a function of wavelength, to be acquired at each pixel in an image, in any imaging mode (transmitted, reflected or fluorescence light, or luminescence). The result is an image cube that contains spectral as well as spatial information. The spectral information can be used to aid segmentation by classifying each pixel in an image according to its spectral signature. Instead of classifying each pixel absolutely by its unique but composite spectrum, it can be modeled as a linear combination of relevant spectra. Given a library of basic spectra known to be an image, the percentage of each basic spectrum contributing to a pixel can be determined. Using these percentages and the total intensity of a pixel, the contribution of each material from the spectral library to a specific pixel can be quantitatively measured. The result is an image cube that contains both spectral and spatial information. Unlike conventional transmittance and fluorescence microscopy, spectral imaging has the ability to generate qualitative and quantitative multiparameter, high-resolution images at the single-cell level. For example, by application of microscopic spectral imaging, we recently documented expression of Stat transcription factors and their down-regulation in individual melanocytes and keratinocytes of atypical nevi obtained from melanoma patients who were treated for 3 months with systemic low-dose interferon-alpha (IFN-α) (10).

To determine whether noninvasive macroscopic spectral imaging can detect melanoma arising in atypical nevi, we imaged 40 atypical nevi in patients with a clinical history of melanoma. One of these atypical nevi, located on a patient's neck, revealed a small and darkly pigmented area close to its border. The lesion was first photographed with a 35 mm camera (Fig. 1A, a and b) and then imaged in vivo (Fig. 1A, c and d). The results of the macroscopic spectral imaging analysis demonstrated a spec-


A
a
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Melanoma
Scar
b
Melanoma

trally segregated area, displayed in pseudocolored blue (Fig. 1A, d), that corresponded to the region of dark pigmentation (Fig. 1A, a and b). Following excision of the nevus with a wide and deep margin, histologic examination of adjacent hematoxylin & eosin (H&E)–counterstained sections of the formalin-fixed and paraffin-embedded specimen revealed the presence of a melanoma in situ (Fig. 1A, f) arising in the atypical nevus (Fig. 1A, e). A year later, macroscopic spectral imaging detected on the back of the same patient (Fig. 1B) spectrally segregated areas (Fig. 1B, b) adjacent to a scar (Fig. 1B, a) that was the result of a previous nevus punch biopsy. As in the previous case, histologic analysis of H&E–counterstained and S-100 antigen–stained sections of the resected tissue (data not shown) revealed the presence of a melanoma in situ in the areas that had been documented by macroscopic spectral imaging to be distinct in their spectral profile from the surrounding tissue.

In another patient who had a large number of atypical nevi and who had undergone surgery for the removal of a VGP melanoma, macroscopic spectral imaging spectrally segregated a small region in an atypical nevus on the patient's leg that was distinct from the surrounding tissue of the nevus. Histopathologic analysis of the excised nevus demonstrated the presence of an RGP melanoma in the area that corresponded to the spectrally segregated region (data not shown).

Of the other 37 atypical nevi that were subjected to macroscopic spectral imaging, none exhibited a spectrally segregated area(s) that was distinct from the macroscopic spectral profile of the entire nevus. Concordant with this finding, histologic examination of H&E–counterstained sections, prepared from each of these atypical nevi following their excision, did not show evidence of a melanoma in any of these atypical nevocytic lesions.

Expression of Molecular and Immunologic Markers in Melanomas In Situ

Over the past decade, a major focus regarding the molecular analysis of advanced-stage melanomas has been the study of genes whose expression is required for the proliferation of VGP and MGP melanomas. The results of these investigations demonstrated that bFGF and one of its receptor, FGFR-1, are expressed in all VGP and MGP melanoma cell lines and specimens analyzed to date, and that expression of this ligand–growth factor receptor pair is essential for the growth of advanced-stage melanomas, both in vitro and in vivo (9,11,12). Furthermore, a molecular study of benign and atypical nevi obtained from patients with a clinical history of melanoma demonstrated expression of bFGF and FGFR-1 in the dermal nevocytes and the stroma of these clinically and histologically distinct types of nevi (8).

Knowing the status of bFGF/FGFR-1 expression in melanoma precursor lesions and advanced-stage melanomas, the purpose of the present study was to determine the status of expression of these two genes in one of the early stages of melanoma development, e.g., in melanoma in situ. Performing in situ hybridization with a bFGF and an FGFR-1 cDNA probe, and immunohistochemistry with a bFGF and human FGFR-1-specific antibody, we analyzed tissue sections prepared from the melanoma in situ that developed in association with the atypical nevus (Fig. 1A) and, as depicted in Figure 2, a melanoma in situ located adjacent to a VGP melanoma. Similar to the findings of the immunohistochemistry analyses (data not shown), the results of the in situ hybridization analyses revealed strong cytoplasmic expression of bFGF and FGFR-1 in the melanoma in situ. In fact, as seen in the case of the melanoma in situ located next to the VGP melanoma (Fig. 3A, B), not only was cytoplasmic staining of bFGF (Fig. 3A, a) and

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Fig. 1. Macroscopic spectral imaging of atypical nevi. (A) Photography, spectral images, and histology of an atypical nevus and a melanoma in situ arising in the nevus. (a, b) 35 mm photographs of the melanocytic lesion are shown. A red-green-blue (RGB) image was derived from the spectral cube of the melanocytic lesion (c). The marked pixels in the RGB image were used to select spectra for classification. In the spectral image (d), the area pseudocolored blue represents the spectrally segregated region containing the melanoma in situ. The H&E–counterstained tissue sections (e) and (f) depict the histology of the atypical nevus (e) and the melanoma in situ (f). (B) RGB and spectral image of a melanoma in situ arising adjacent to a scar from a nevus punch biopsy. An RGB image (a) and a pseudocolored macroscopic spectral image (b) show an area containing a scar and adjacent to it, a melanoma in situ. In the spectral image (b), the spectrally segregated regions assigned the pseudocolor green correspond to tissue sections that were diagnosed by histopathology to contain melanoma in situ.

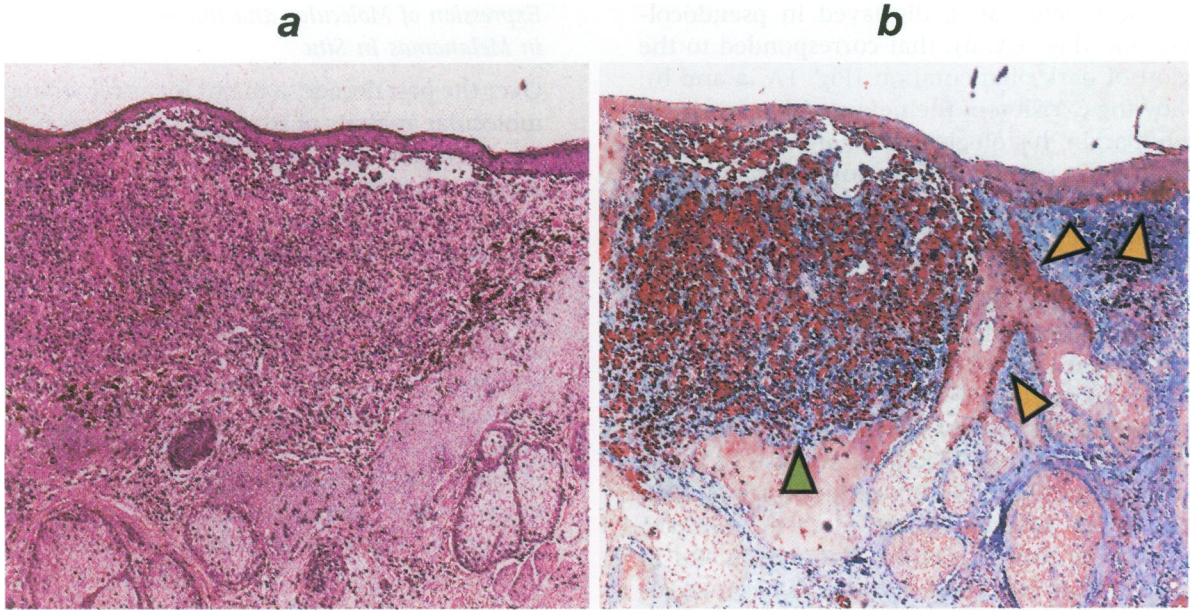

Fig. 2. RGB images of H&E– and S100 antigen-stained sections of a melanoma in situ and an adjacent VGP melanoma. (a) H&E–stained section prepared from a formalin-fixed and paraffin-embedded melanoma in situ and an adjacent VGP melanoma is shown. The yellow-colored arrows in the S-100 antigen–stained tissue section (b) point to the melanoma in situ, and the green-colored arrow points to the adjacent VGP melanoma.

FGFR-1 (Fig. 3B, a) detected in every cell constituting the melanoma in situ but the degree of bFGF/FGFR-1 expression in these cells was comparable to that detected in the cells of the adjacent VGP melanoma (Fig. 3A, b and 3B, b). The skin surrounding the melanomas (Fig. 3A, c and 3B, c) revealed expression of bFGF (Fig. 3A, c) and FGFR-1 (Fig. 3B, c) predominantly in cells comprising the basal layer.

The two major functions of adhesion molecules are to facilitate cell–cell contact and attach cells to the extracellular matrix (ECM). In addition, in tumor development, adhesion molecules play an important role in cell migration, proliferation, differentiation, apoptosis, and immune recognition (13). A family of adhesion molecules whose expression changes with progression to advanced-stage melanomas are the homophilic cadherins. For example, normal melanocytes express E-cadherin, which mediates their adhesion to the surrounding keratinocytes (14). In contrast, in vitro and in vivo, expression of E-cadherin in VGP and MGP melanomas appears to be down-regulated (14,15), raising the possibility that concomitant with melanocytic progression, loss of E-cadherin expression may help sever adhesion of the melanocytic cells from their surrounding keratinocytes. While E-cadherin is differentially expressed in normal melanocytes as compared to VGP and MGP melanomas, the status of N-cadherin expression in the different stages of melanocytic progression is less clear.

As depicted in Figure 4, expression of N-cadherin was detected in almost every cell comprising the melanoma in situ (Fig. 4a) and the adjacent VGP melanoma (Fig. 4b). In contrast, immunohistochemical analysis of four different MGP melanoma specimens revealed a heterogeneous pattern of N-cadherin expression. One of the four MGP melanoma specimens revealed expression of N-cadherin in 80% of its cells. The second one demonstrated N-cadherin staining in approximately 50% of its cells, and the third and fourth MGP melanoma specimen (Fig. 4c) exhibited about 10% staining. In the skin surrounding the four MPG melanomas, expression of N-cadherin was almost exclusively detected in epidermal keratinocytes (data not shown). Similarly, a total of 15 benign and atypical nevi that were probed with the same N-cadherin-specific antibody showed expression of N-cadherin predominantly, if not exclusively, in the keratinocytes of the epidermal compartment (data not shown). Parallel to these studies, we also probed sections of the same skin, nevus, and melanoma specimens with an antibody to the cell–cell adhesion molecule, E-cadherin. Strong expression of E-cadherin was detected in epidermal keratinocytes, to a lesser extent in the epidermal–dermal junction of the benign and atypical nevi, and in

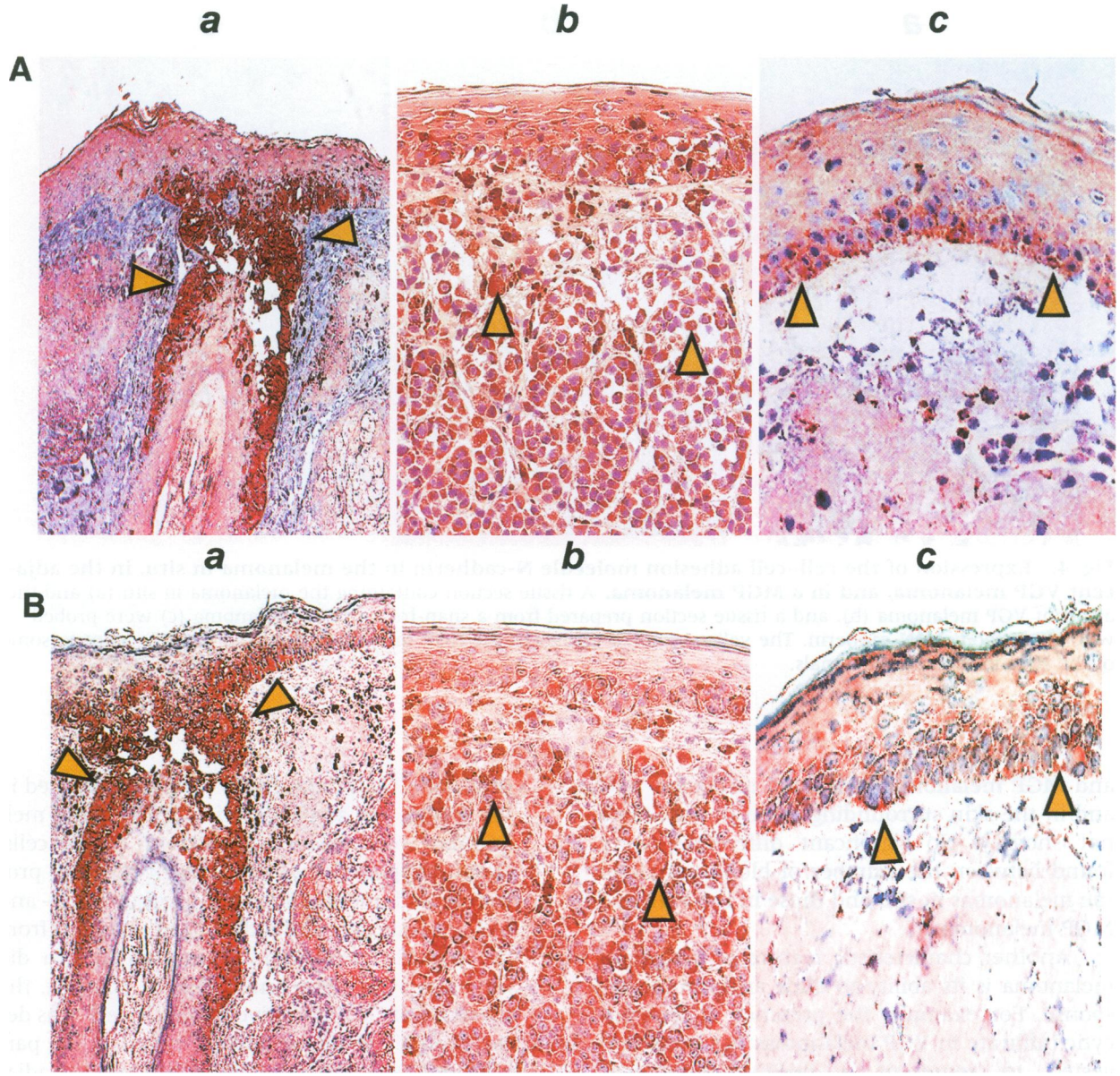

Fig. 3. Expression of bFGF and FGFR-1 in the melanoma in situ, in the adjacent VGP melanoma, and in skin surrounding the melanomas. Tissue sections containing the melanoma in situ (a), the VGP melanoma (b), and skin adjacent to the melanomas (c) were analyzed by in situ hybridization using a human bFGF-specific (A) and a human FGFR-1-specific (B) cDNA probe. The yellow-colored arrows in the red-green-blue (RGB) images of the tissue sections point to some of the bFGF- and FGFR-1-specific hybridization signals.

the skin surrounding the melanomas. In contrast, the melanoma in situ and VGP and MGP melanoma specimens exhibited little or no staining (data not shown). Thus, our findings are in agreement with the results of previous studies (14,15), which documented down-regulation of E-cadherin concordant with progression to advanced-stage melanomas.

Among solid malignancies, melanoma, in particular, MGP melanoma, represents one of the most vascularized tumors. To determine whether melanomas in situ compared to VGP and MGP melanomas exhibited differences in the number of blood vessels interspersing and surrounding the tumors, we probed sections from the different tumors and surrounding skin with an antibody to CD31 antigen, which is expressed on vascular endothelial cells (16). The results of this immunohistochemical analysis demonstrated significantly more blood vessels in VGP

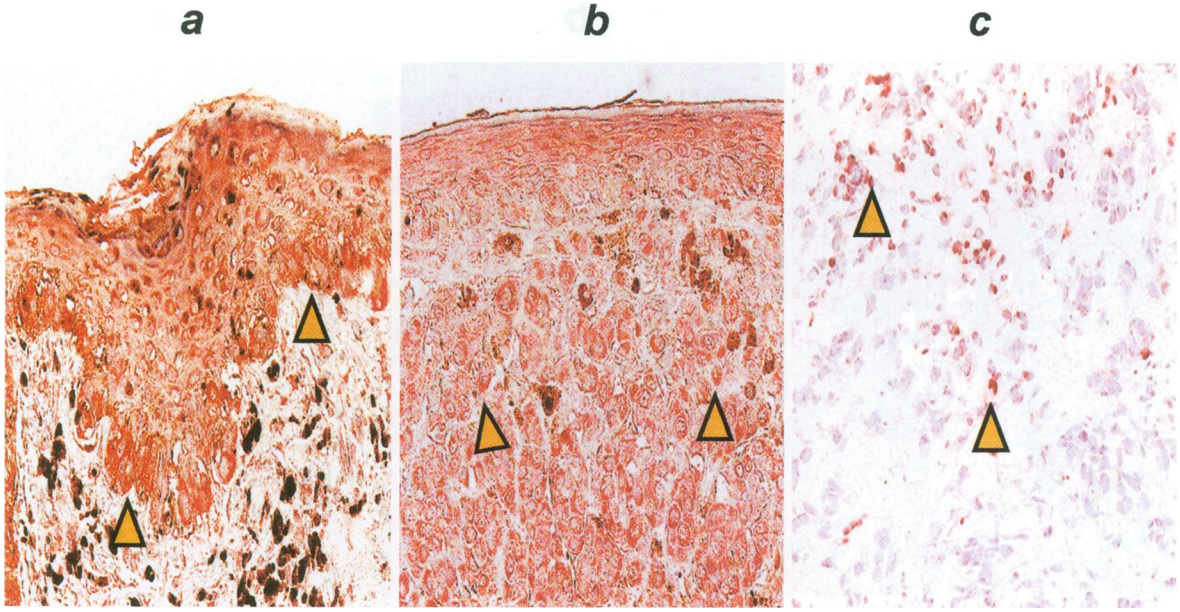

Fig. 4. Expression of the cell–cell adhesion molecule N-cadherin in the melanoma in situ, in the adjacent VGP melanoma, and in a MGP melanoma. A tissue section containing the melanoma in situ (a) and the adjacent VGP melanoma (b), and a tissue section prepared from a snap-frozen MGP melanoma (c) were probed with an antibody to N-cadherin. The yellow-colored arrows in the RGB images of the tissue sections point to some of the N-cadherin-specific signals.

and MGP melanomas compared to benign nevi and in the skin surrounding these tumors (data not shown). No significant differences were found between the number of blood vessels in the melanomas in situ and those in the VGP and MGP melanomas.

Another characteristic feature of malignant melanoma is its ability to elicit an immune response. For example, the presence of lymphocytic infiltrate in VGP melanomas has been suggested to represent a positive prognostic indicator (17). To assess the presence or absence of T cells, the melanoma peptide antigen MAGE-1 (18), and antigen-presenting dendritic cells in the melanomas in situ and in VGP and MGP melanomas, we performed immunohistochemistry with antibodies to each of these immunologic markers. Although absent in some but not all tissue sections prepared from different biopsy specimens representing normal skin, CD3-positive T cells were detected in the benign and atypical nevi as well as in the MGP melanomas, with little or no quantitative differences between the nevus and melanoma specimens. Similarly, although MAGE-1 was not detected in normal skin, it was present in the benign and atypical nevi and registered very strongly in the MGP melanomas. In contrast, CD8-positive T cells and CD4 T helper cells were not detected in the benign and atypical nevi. Only the MGP melanomas were positive for CD4 T helper cells. Despite application of the antigen retrieval procedure and use of different sources of T cell– and MAGE-1–specific antibodies, tissue sections from the paraffin-embedded melanomas in situ did not react with these antibodies. In contrast, the presence of CD83-positive dendritic cells was detected in all of the specimens, including the paraffin-embedded melanomas in situ and the adjacent VGP melanoma. Although the number of CD83-positive dendritic cells varied somewhat from specimen to specimen, significant quantitative differences that might have suggested a correlation between increasing numbers of dendritic cells and progression from atypical nevi to MGP melanoma were not apparent.

Discussion

Two of the central questions concerning early detection and molecular/immunologic analysis of malignant melanoma are: what types of new optical imaging techniques/devices can be implemented in the clinic to detect melanoma in its early stages of development, and at what stage in

the progression pathway of melanoma are genes that play an important role in the late stages of the malignancy activated and expressed?

Early detection of premalignant and malignant lesions and visualization of disease progression and treatment efficacy are some of the challenges presented by the clinical application of new and advanced optical imaging techniques. Capitalizing on the intrinsic optical properties of biological tissues, such as light scattering, absorption, polarization, and autofluorescence, investigators have used noninvasive optical imaging techniques such as time-resolved and phase-modulation spectroscopy to quantify hemoglobin saturation in tissue (19) and detect head and neck, bladder, and breast tumors (20). Confocal and multiphoton excitation scanning laser microscopy have been used to visualize cellular and subcellular structures of tissues and organs (21,22).

One of the major difficulties with noninvasive optical imaging of pigmented tissue, such as the skin or skin cancers, in particular melanoma, is the high scattering in the visible and near-infrared range, which significantly reduces image contrast and resolution. Two advanced optical imaging techniques that have yielded the most promising results for visualizing morphological details in normal skin and diagnosing pathological alterations in skin lesions are optical coherence tomography (OCT) (23,24) and spectral imaging, respectively.

We recently demonstrated the capability of microscopic spectral imaging to document the biological impact of low-dose systemic IFN-treatment on distinct members of the family of Stat transcription factors in melanoma precursor lesions (10). We now provide the first examples documenting that macroscopic spectral imaging has the capability to detect in vivo melanoma arising in melanocytic precursor lesions. Given these findings, we believe that macroscopic spectral imaging will be one of the optical imaging techniques that, in light of the rising incidence of melanoma worldwide, can effectively address the need for noninvasive characterization of atypical melanocytic lesions at first presentation, i.e., prior to their excision and histologic analysis.

The second and equally important aim of our investigation was to determine whether melanomas in situ compared to VGP and MGP melanomas exhibit differences with respect to molecular, vascular, and immunologic markers. The results of these analyses provided a first indication that bFGF–FGFR-1, a growth factor–growth factor receptor pair whose expression is required for the proliferation of VGP and MGP melanomas (9,11,12), is strongly expressed in melanomas in situ. Likewise, the adhesion molecule N-cadherin, which sustains the cell–cell adhesion of VGP and MGP melanomas, is strongly expressed in melanomas in situ. Furthermore, the number of blood vessels and dendritic cells detected in the melanomas in situ was comparable to the number of blood vessels and dendritic cells seen in the VGP and MGP melanomas examined in this study.

Although it is possible that the findings reported here are limited to the melanomas in situ evaluated in this study and may not be representative of all melanomas in situ, they nevertheless raise an interesting question: are expression and presence of these known genes and antigens as essential for the biological functions of melanomas in situ as they are for VGP and MGP melanomas, or are there other and perhaps unknown genes that govern the biological properties of melanomas in situ, and is it the dysfunction of these genes that is responsible for the progression to advanced-stage melanomas? Since it has not been possible thus far to conduct in vitro studies with cell cultures representing melanoma in situ, the first part of the question remains unanswered. The second part of this question is being addressed by scientific efforts to isolate genes that are differentially expressed in the early, compared to the late, stages of melanoma.

Acknowledgments

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