Expression of the Tumor Suppressor Gene Product p16\(^{\text{INK4}}\) in Benign and Malignant Melanocytic Lesions

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The gene MTS1 encodes p16\(^{\text{INK4}}\), an inhibitor of cyclin-dependent kinase 4, and is frequently deleted, mutated, or silenced by promoter methylation in melanoma cells and in the germline of familial melanoma patients. Although MTS1 may thus be the candidate melanoma suppressor gene that maps to chromosome 9p21, it is not clear how dysfunction at that locus temporally relates to melanoma progression. To further test its role in sporadic melanoma, the expression of p16\(^{\text{INK4}}\)-protein and -mRNA was characterized in melanomas and melanocytic nevi by immunocytochemistry and in situ reverse transcriptase-polymerase chain reaction. Histologic tissue sections were immunolabeled with anti-p16\(^{\text{INK4}}\) antibody for 108 melanocytic lesions, including common and atypical nevi, in situ melanomas, primary invasive melanomas, and metastatic tumors. A subset of the lesions was analyzed for expression of p16\(^{\text{INK4}}\)-mRNA, employing forward and reverse intron-bridging primers for reverse transcriptase-polymerase chain reaction amplification of the transcript corresponding to exons 1 and 2 of MTS1.

Strong immunolabeling was detected in the melanocytes of common nevi and of nevi with architectural disorder, cytologic atypia. By digital image analysis, in contrast, labeling intensity decreased significantly and progressively in the melanocytes of in situ, invasive, and metastatic melanomas. Results from the in situ reverse transcriptase-polymerase chain reaction analysis were confirmatory, showing a strong signal in the melanocytic nevi but progressive signal attenuation with increasing stage of melanoma. These data indicate correlation between gradual loss of expression of the MTS1 locus and progression of melanoma, further supporting an emerging role for the gene in the malignant transformation of melanocytes. The failure to demonstrate reduced expression in nevi suggests either that these lesions are not an early stage in melanoma development, in contrast to prevailing assumptions, or that loss of p16\(^{\text{INK4}}\) function is not an initiating event in melanocyte transformation.


C onvergent evidence from deletional mapping of melanoma tumor cells (Fountain et al., 1992) and genetic linkage analyses of melanoma kindreds (Cannon-Albright et al., 1992) has assigned a melanoma susceptibility locus (MLM) to chromosome 9p21. Positional cloning of the smallest region commonly deleted homozygously in melanoma cell lines later identified a gene, multiple tumor suppressor locus (MTS1), as the candidate locus for MLM (Kamb et al., 1994a; Nobori et al., 1994). The cDNA coding sequence of the expressed protein, p16\(^{\text{INK4}}\), had previously been identified with two-hybrid screening by investigators searching for natural inhibitors of the cyclin dependent kinases, which are molecules that enable passage of cells through G1-S cell cycle checkpoints (Serrano et al., 1993). Subsequently, observations of segregating germine mutations in melanoma kindreds (Hussussian et al., 1994; Kamb et al., 1994b) and of somatic mutations in tumor cell lines of multiple histogenetic lineages (Smith-Sørensen and Hovig, 1996) have strongly supported the candidacy of MTS1 as a general tumor suppressor locus for common cancers and more specifically for familial and sporadic melanoma.

Although inactivation of the MTS1 locus in cancer cells fits well conceptually with its candidate role as a tumor suppressor gene, given the apparently critical inhibitory function of p16\(^{\text{INK4}}\) in cell cycle regulation, the temporal relationship of mutational lesions in the gene to the initiation and progression of melanoma has not been elucidated. It is generally acknowledged that, in accordance with the theory of step-wise transformation, melanomas may evolve from melanocytic nevi as precursor lesions having undergone the first stage(s) in the neoplastic process (Clark et al., 1984; Elder et al., 1989). Indeed, the spatial coexistence of incident melanomas with remnants of melanocytic nevi observed in histologic sections by numerous studies, tends to support this assertion (Skender-Kalnenas et al., 1995), as do observations of malignant degeneration in congenital nevi and the direct correlation between total numbers of nevi and relative risk for melanoma in the case-control literature (reviewed by Piepkorn, 1994). It is reasonable to expect, therefore, that the genome of nevus cells will harbor the molecular alterations necessary for the initiation of melanoma. Accordingly, we postulated that, given the strong case for MTS1 as a major locus contributing to the development of melanoma, abnormalities in expression of the gene at the mRNA or protein level will be demonstrable in the cells of melanocytic nevi. The studies presented herein represent a test of this specific hypothesis and of the more general hypothesis that inactivation of MTS1 correlates with progression of melanoma. Furthermore, in view of the suggestion that expression

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Abbreviations: cKGM, keratinocyte growth medium; cMGM, melanocyte growth medium.
of p16\(\text{INK4}^\text{a}\) and the retinoblastoma gene product may be inversely correlated in some tumor systems (Otterson et al., 1994), the status of RB1 expression was compared with that of p16\(\text{INK4}^\text{a}\) in a subset of the lesions. Our results extend those of Reed et al. (1995) and Talve et al. (1997), who immunolabeled melanocytic lesions with antibodies against p16\(\text{INK4}^\text{a}\). In qualitative analyses, Reed et al. (1995) observed decreased labeling in invasive and metastatic melanomas, but uniform labeling in melanocytic nevi and in situ melanomas, suggesting that loss of p16\(\text{INK4}^\text{a}\) expression may not be an initiating event in transformation. Talve et al. (1997) confirmed both the uniform labeling of nevus cells and the loss of expression with melanoma progression, but they observed decreased expression in melanoma in situ. Neither study specifically evaluated atypical (dysplastic) nevi. As reported herein, our observations support the findings of Talve et al. (1997) that p16\(\text{INK4}^\text{a}\) immunostaining is reduced in some melanomas in situ, which is consistent with the locus being an early target in melanoma initiation. The original aspects of our report include: quantitation of p16\(\text{INK4}^\text{a}\) immunostains of melanocytic tumors by digital image analysis; the observation of normal levels of p16\(\text{INK4}^\text{a}\) immunolabeling in atypical nevi, which is notable because such lesions are purported to be melanoma precursor lesions; the lack of an apparent inverse correlation between p16\(\text{INK4}^\text{a}\) and RB1 expression in melanomas; and the finding that expression levels of p16\(\text{INK4}^\text{a}\) mRNA by in situ reverse transcriptase polymerase chain reaction (RT-PCR) correlate with reductions in p16\(\text{INK4}^\text{a}\) immunolabeling during melanoma progression.

**MATERIALS AND METHODS**

**Cell culture** Primary keratinocyte and melanocyte cultures were established from human neonatal foreskins (Fugger et al., 1992; Pepkorns et al., 1994) and grown in complete keratinocyte growth medium (CKGM; Clonetics, San Diego, CA), or complete melanocyte growth medium (CMGM; Clonetics), respectively. The cells were used at the second to fourth passage levels. Human melanoma cell lines HTB 66, HTB 70, and HTB 71, and a human breast cancer cell line, MCF7, were obtained from the American Type Culture Collection (Rockville, MD) and grown as monolayered cultures in Dulbecco’s minimum essential medium (BioWhittaker, Walkersville, MD), containing 10% fetal bovine serum (Intergen, Purchase, NY). Two days prior to the immunolocalization experiments, cells of each cell type were subcultured into LabTek chamber slides (Nunc, Naperville, IL) at 1–2 \(10^4\) cells per cm\(^2\).

**Tissue samples** Five micrometer recuts were prepared from formalin-fixed, paraffin-embedded tissues retrieved from the pathology files of the University of Washington Medical Center. The series of formalin-fixed lesions, including common and atypical nevi, in situ melanomas, primary invasive melanomas, and metastatic tumors. The original diagnoses were confirmed by review of the hematoxylin and eosin stained sections using standard diagnostic criteria for each lesion type as applied by one of us (MP). The diagnoses of the metastatic lesions were corroborated by review of the routine histology and immunoperoxidase studies with markers for S100 protein and/or HMB-45.

**Western immunoblotting** The specificities of the anti-p16\(\text{INK4}^\text{a}\) mouse monoclonal antibody, G175–405, and the anti-retinoblastoma protein (RB1) monoclonal antibody, G3–245 (both purchased from PharMingen, San Diego, CA), were established by western immunoblotting of cell extracts from human neonatal keratinocyte and melanocyte cultures, established melanoma cell lines, and a breast cancer cell line (MCF 7). Cell layers in exponential growth were washed twice with chilled phosphate-buffered saline and scraped into 300 µl of cold lysis buffer (50 mM Tris HCl, 0.25 M NaCl, 0.1% Triton X-100, 1.0 mM ethylenediaminetetraacetic acid, 50 mM NaF, 1.0 mM diithiothreitol, 0.1 mM Na\(_2\)VO\(_4\)) with 0.1 mM phenylmethanesulfonyl fluoride, 1 µg leupeptin per ml, 10 µg soybean trypsin inhibitor per ml, 10 µg N-tosyl-L-lysine chloromethyl ketone per ml, 10 µg N-p-tosyl-L-tyrosine chloromethyl ketone per ml, and 1 µg aprotinin per ml (Tan et al., 1994). Following incubation on ice for 30 min, the cells were pooled and homogenized, and the homogenates were centrifuged for 5 min at 300 \(\times\) g at 4°C. The protein concentrations in the supernatants were measured with the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA), and the supernatants were concentrated into sample buffer. Twenty-five to 50 µg of total protein from each cell extract were resolved electrophoretically by nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis, along with molecular-weight markers (Amersham, Arlington Heights, IL; Gibco BRL, Gaithersburg, MD; and Novex, San Diego, CA) using 12% and 7.5% acrylamide gels. The gels were transferred for 1 h at 100 V to nitrocellulose sheets (BioRad), which were blocked with Tris-saline (50 mM Tris base, 0.15 M NaCl, pH 7.4) containing 0.25% gelatin.

**Immunostaining** In preliminary analyses, the G175–405 anti-p16\(\text{INK4}^\text{a}\) monoclonal antibody established by western immunoblotting of cell extracts from keratinocytes and melanocytes, melanoma lines, and the MCF 7 mammary carcinoma line. The keratinocyte, melanocyte, and HTB 66 extracts have been resolved in lanes 4, 6, and 8 and the p16\(\text{INK4}^\text{a}\) negative cell lines (HTB 70, HTB 71, and MCF 7) in lanes 2, 3, and 7, respectively. Molecular weight markers are represented in the first and fifth lane. In addition to p16\(\text{INK4}^\text{a}\), G175–405 also detected a faint, uncharacterized band at \(\sim 27\) kDa, which others have previously observed with this and other anti-p16\(\text{INK4}^\text{a}\) antibodies. The molecular weight of the standard marker is indicated in the left margin. Detection was by enhanced chemiluminescence.

0.05% NP-40 (Sigma, St. Louis, MO)/5 mM ethylenediaminetetraacetic acid for 1 h at room temperature and incubated with a 1:250 dilution of the primary anti-p16\(\text{INK4}^\text{a}\) antibody or anti-RB1 antibody stock solution (0.5 mg per ml) overnight at 4°C. The blots were washed with buffer three times and incubated with 1:1000 secondary, horse anti-mouse antibody conjugated to horseradish peroxidase (Vector Laboratories, Burlingame, CA) for 60 min at room temperature. Following a wash with 0.25% gelatin/0.05% NP-40/0.4% sarcosyl in Tris-saline, immunolocalization was by enhanced chemiluminescence according to the manufacturer (ECL, Amersham) with 3 to 40 s exposure times (see Fig. 1).

**Image analysis** Immunoperoxidase labeling intensities were quantitated with an image analysis system based on Optimas 4.0 image analysis software (Optimas, Bothell, WA), using a Zeiss Universal microscope equipped with epi-illumination or with a Bio-Rad MRC-600 laser scanning confocal microscope.
uniform labeling. To localize p16INK4-specific mRNA sequences within melanocytic lesions, Five RT-PCR
in situ intensities were compared. In staining was reduced by batch processing of those lesions for which labeling
exhibited greater variability of staining between cells (not shown). Variability similar for the two sampling strategies but the randomly selected cells generally
lesions were also analyzed using manual sampling. Average cell labeling was
sampling from computer generated coordinates. For comparison, slides of other
of these cells within the epidermal keratinocytes did not lend itself to random
selecting representative neoplastic melanocytes because the pagetoid distribution
of lesions

<table>
<thead>
<tr>
<th>Primers</th>
<th>Position</th>
<th>Annealing temperature</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sense</td>
<td>5’TACGGTCCGAGGCCGATCCAGGTC</td>
<td>170–193</td>
<td>336</td>
</tr>
<tr>
<td>Anti-sense</td>
<td>5’GGGAATGTCCTGAGGGACCTTCCGC</td>
<td>482–505</td>
<td></td>
</tr>
</tbody>
</table>

*To reduce the generation of PCR products from genomic DNA, intron bridging primers were designed.

selecting representative neoplastic melanocytes because the pagetoid distribution of these cells within the epidermal keratinocytes did not lend itself to random sampling from computer generated coordinates. For comparison, slides of other lesions were also analyzed using manual sampling. Average cell labeling was similar for the two sampling strategies but the randomly selected cells generally exhibited greater variability of staining between cells (not shown). Variability in staining was reduced by batch processing of those lesions for which labeling intensities were compared.

In situ RT-PCR. A four-step protocol described by Nuovo (1994) was used to localize p16INK4-specific mRNA sequences within melanocytic lesions. Five micrometer thick paraffin-sections of formalin-fixed tissue were transferred to silane-coated slides, dewaxed, and protease digested to facilitate reagent penetration into the cells. The sections were incubated with pepsin (Sigma) at 2 mg per ml for varying incubations (15–90 min), as adjusted for the estimated lengths of time the tissues had been fixed in formalin. The KT and PCR steps were performed in a GeneAmp In Situ PCR System 1000 (Perkin-Elmer, Foster City, CA). After digestion with 10 U per tissue section of RNase-free DNase (Boehringer Mannheim, Indianapolis, IN), to block amplification of genomic DNA, the mRNA was reverse-transcribed to generate cDNA templates by SuperScript S2 Reverse Transcriptase (BRL, Gaithersburg, MD), according to the manufacturer’s specifications. For PCR amplification of the cDNA and direct labeling of the amplified product, the following mixture was used: 2.5 mM MgCl$_2$, 200 µM cocktail of dNTP, 10 µM digoxigenin-11-2’-deoxyuridinyl-5’-triphosphate (Boehringer Mannheim), 0.8 µM forward and reverse primers (see Table I), 0.08% bovine serum albumin (Sigma), and 2.5 U Taq DNA Polymerase (BRL). Synchronized “hot start” PCR was achieved using the temperature-sensitive Taq-blocking monoclonal antibody supplied by Clontech (Palo Alto, CA) (Kellogg et al., 1994; Martinez et al., 1995). A 25 µl aliquot of solution was applied to each section and 20 thermal cycles proved adequate to obtain crisp staining. Detection of the digoxigenin-labeled product utilized a kit from Boehringer Mannheim, which employed an anti-digoxigenin alkaline phosphate-conjugated antibody, followed by exposure to nitroblue tetrazolium chloride and 5-bromo-chloro-3-indolyl-phosphate. The dark-purple reaction product was visualized by bright-field microscopy.

Negative (DNase-digestion, no reverse transcriptase) and positive controls (no DNase-digestion) were performed on the same glass slide with each test. Before the final in situ RT-PCR analyses, all parameters for the PCR reaction, including MgCl$_2$ concentration, pH, and annealing temperature, had been optimized by standard solution phase PCR.

RESULTS

Specificities of the anti-p16INK4, G175–405, and the anti-RB1, G3–245, monoclonal antibodies established by western immunoblotting The specificities of the p16INK4 and RB1 antibodies were evaluated by western immunoblotting of cell extracts from human neonatal keratinocyte and melanocyte cultures, established melanoma cell lines, and a breast cancer cell line (MCF 7). A full-length recombinant bacterially produced GST-p16INK4 fusion protein was used as immunogen for preparation of the anti-p16INK4 antibody (Serrano et al., 1993). A major band migrating with an apparent mass of ≈16 kDa was detected from keratinocyte.

Table II. p16INK4 is differentially expressed in benign and malignant melanocytic lesions

<table>
<thead>
<tr>
<th>Expression level</th>
<th>Melanocytic nevi</th>
<th>Melanoma in situ</th>
<th>1° Invasive melanoma</th>
<th>Metastatic melanoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1 (3%)</td>
</tr>
<tr>
<td>0–1+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1 (3%)</td>
</tr>
<tr>
<td>1+</td>
<td>1 (2%)</td>
<td>–</td>
<td>3 (18%)</td>
<td>4 (12%)</td>
</tr>
<tr>
<td>1–2+</td>
<td>1 (2%)</td>
<td>–</td>
<td>–</td>
<td>1 (3%)</td>
</tr>
<tr>
<td>2+</td>
<td>5 (10%)</td>
<td>1 (14%)</td>
<td>3 (18%)</td>
<td>7 (21%)</td>
</tr>
<tr>
<td>2–3+</td>
<td>–</td>
<td>1 (14%)</td>
<td>1 (6%)</td>
<td>4 (12%)</td>
</tr>
<tr>
<td>3+</td>
<td>44 (86%)</td>
<td>5 (72%)</td>
<td>10 (50%)</td>
<td>16 (49%)</td>
</tr>
<tr>
<td>Number of lesions</td>
<td>51</td>
<td>7</td>
<td>17</td>
<td>33</td>
</tr>
</tbody>
</table>

*0, nonreactive; 1+, less than one-third of melanocytic cells showing uniform labeling; 2+, one- to two-thirds showing uniform labeling; 3+, at least two-thirds showing uniform labeling.
Table III. RB1 status is independent of p16INK4 expression in melanocytic lesions

<table>
<thead>
<tr>
<th>Expression level</th>
<th>RB1 &lt; 30%a</th>
<th>RB1 ≥ 30%a</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>p16INK4 0–2+</td>
<td>4 (14%)</td>
<td>7 (25%)</td>
<td>11</td>
</tr>
<tr>
<td>p16INK4 &gt;2+</td>
<td>2 (7%)</td>
<td>15 (54%)</td>
<td>17</td>
</tr>
<tr>
<td>Total</td>
<td>6</td>
<td>22</td>
<td>28b</td>
</tr>
</tbody>
</table>

aProportion of cells with nuclear labeling.
bn = 28 (four nevi, three atypical nevi, three melanomas in situ, seven primary invasive melanomas, 11 metastatic melanomas).

Subcellular localization of immunolabeled p16INK4 and RB1 imaged by confocal microscopy For localization of p16INK4 and RB1 proteins by immunofluorescence, cultures of human neonatal keratinocytes and melanocytes, established melanoma cell lines, and a breast cancer cell line (MCF 7) were immunolabeled with anti-p16INK4 or anti-RB1 antibody and detected with fluorescein isothiocyanate- or Texas Red-conjugated secondary antibodies. Subcellular localization was imaged by scanning laser confocal microscopy, and the results for the HTB 66 and HTB 70 cell lines are illustrated in Fig 2. Immunolabeled p16INK4 displayed diffusely granular cytoplasmic fluorescence, with random perinuclear and focal granular nuclear staining, in melanocytes, keratinocytes, and the HTB 66 melanoma cell line (Fig 2A). In contrast, there was no detectable immunolabeling of the p16INK4-negative cell lines MCF 7, HTB 71, and HTB 70 (Fig 2C). Reed et al (1995) also reported combined cytoplasmic and nuclear staining by immunoperoxidase labeling of melanocytic cells with a polyclonal anti-p16INK4 antibody.

Immunolabeling with anti-RB1 demonstrated granular nuclear, but minimal cytoplasmic, staining for all tested cell types, as illustrated in Fig 2B. D) for HTB 66 and HTB 70, respectively.

Immunolabeling patterns for p16INK4 and RB1 in melanocytic lesions are distinct Immunoperoxidase staining for p16INK4 and RB1 was performed on histologic sections from benign and malignant melanocytic lesions. Keratinocytes, which were found in these studies to strongly express p16INK4, served as positive internal controls for those lesions present within skin. The slides were reviewed independently by two observers; the semiquantitative visual scores are summarized in Table II. Uniform labeling in at least two-thirds of melanocytic cells was detected in 86% of common and atypical melanocytic nevi (Fig 3A, B), labeling at this proportion of the cell populations decreased to 72% for melanomas in situ (Fig 3C) and to 59% for primary invasive melanomas, whereas only 49% of metastatic tumors expressed p16INK4 at this level (Fig 3D). p16INK4 labeling thus attenuated with advancement in tumor stage, as referenced to the consistent staining of keratinocytes as the internal control. Because it has been suggested that p16INK4 and RB1 expression may be inversely correlated in some tumor systems (Otterson et al, 1994), and because nuclear expression of RB1 has been reported to be higher in melanomas than in nevi (Saenz-Santamaria et al, 1995), immunohistochemistry for RB1 was also performed in about one-third of the melanocytic lesions that had been immunolabeled for p16INK4 (Table III). No inverse correlation between the RB1 status and p16INK4 expression was observed in this series. Strong labeling for p16INK4 and RB1 was detected in 54% of the melanocytic lesions, and only 7% strongly expressed p16INK4 when RB1 staining was minimal to negative. A similar absence of inverse correlation between the expression levels of p16INK4 and RB1 by immunolabeling has been reported in nonmelanoma malignancies (Geradts et al, 1995).

Intensity of p16INK4 immunolabeling quantitatively attenuates with melanoma progression Histologic slides immunostained for p16INK4 were quantitated for labeling intensity with the Optimas image analysis system (Materials and Methods). The results are presented in Fig 4. In seven cases of melanomas with coexisting melanocytic nevi, the nevus cells strongly expressed p16INK4, whereas adjacent melanoma cells consistently displayed reduced staining intensity, and the keratinocytes expressed p16INK4 at an intermediate level (Fig 4A). Quantitative comparisons of the levels of p16INK4 within a lymph node.
primary invasive melanomas, and metastatic melanomas indicated strong immunostaining intensities in common and atypical nevi, melanomas in situ.

The mean for common nevi at p<0.05, melanoma, and metastatic melanoma are significantly decreased compared with melanoma. Numbers of lesions are given in parentheses.

Atypical nevus 15 0 2 13 0 4 11

1

Melanoma:

1

In situ

1

4 0 1 3 0 2 2

1

Invasive

6 0 1 5 0 4 2

1

Metastatic

13 4 4 5 3 4 6

1

Table IV. p16INK4 mRNA expression in melanocytic lesions correlates with p16INK4 immunolabeling

<table>
<thead>
<tr>
<th>Lesion type</th>
<th>Number</th>
<th>Protein expression level</th>
<th>mRNA expression level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nevus</td>
<td>23</td>
<td>1+ 0 1 22 0 5 18</td>
<td></td>
</tr>
<tr>
<td>Atypical nevus</td>
<td>15</td>
<td>0 2 13 0 4 11</td>
<td></td>
</tr>
<tr>
<td>Melanoma: in situ</td>
<td>4</td>
<td>0 1 3 0 2 2</td>
<td></td>
</tr>
<tr>
<td>Invasive</td>
<td>6</td>
<td>0 1 5 0 4 2</td>
<td></td>
</tr>
<tr>
<td>Metastatic</td>
<td>13</td>
<td>4 4 5 3 4 6</td>
<td></td>
</tr>
</tbody>
</table>

*1+, less than one-third of melanocytic cells with uniform labeling; 2+, one- to two-thirds with uniform labeling; 3+, at least two-thirds with uniform labeling.

DISCUSSION

With these studies we have further evaluated the chromosome 9p21 locus, MTS1, as a candidate melanoma suppressor gene in a series of melanocytic lesions ranging from common nevi to metastatic melanoma. Immunolabeling of the expressed product, p16INK4, was correlated with analysis for p16INK4-mRNA by in situ RT-PCR with intron bridging primers and was compared with the immunolabeling status of RB1 in a representative subset of the lesions. The specificity of the p16INK4 monoclonal antibody used for these purposes was corroborated by western immunoblotting of normal and transformed cell lines, including cells that do not express p16INK4 (Fig 1). Imaging of immunolabeled p16INK4 by scanning laser confocal microscopy indicated that the protein is distributed to both the cytoplasms and the nuclei of normal and transformed melanocytes, in agreement with previous studies (Reed et al, 1995; Talve et al, 1997), whereas RB1 was generally restricted to the nuclei (Fig 2). No inverse correlation was apparent between the expression status of RB1 and p16INK4 in these melanocytic lesions, contrary to expectations. Moreover, expression levels of p16INK4 protein and -mRNA in the nevomelanocytes of both common and atypical (dysplastic) nevi were not appreciably reduced. The transformed melanocytes of in situ, invasive, and metastatic melanomas, however, expressed p16INK4 at significantly reduced levels by quantitative immunolabeling (Fig 4), and an inverse correlation between p16INK4-protein and -mRNA levels and stage of the disease was observed, suggesting a role in the progression of melanoma.

Current understanding of neoplastic transformation invokes a stepwise pathway of progression to the fully malignant phenotype in tumor systems. This paradigm has been best characterized from studies of colon carcinoma cells (Powell et al, 1992), but it may be generally applicable to many tumor systems, including melanoma (Elder et al, 1989). Under the general model of melanoma progression, certain morphologic variants of melanocytic nevi have been incorporated as a first stage in carcinogenesis (Clark et al, 1984; Elder et al, 1989). It is reasonable, therefore, to evaluate these putative precursor lesions for molecular alterations in the search for the events initiating transformation to melanoma.

The familial melanoma locus, MTS1, is a plausible target for a genetic lesion that initiates transformation. Data acquired from the genetic linkage of MTS1 markers at chromosome 9p21 to familial melanoma (Cannon-Albright et al, 1992) and from deletional mapping of melanoma tumors, which has shown chromosome 9p21 to be the most common genomic target for loss of heterozygosity and homozygous deletions in some analyses (Fountain et al, 1992), are compatible with a role in tumor initiation. The alternative hypothesis of a more distal event in progression is counterintuitive, unless another covert initiating mutation depends on coordinate,
distal inactivation of MTS1 for clinically overt disease in the families that were used for genetic linkage.

Our data are consistent with an important role for p16\textsuperscript{INK4} inactivation during melanoma progression, regardless of the actual chronology. A perceivable decrease in the expression level of p16\textsuperscript{INK4}-protein and -mRNA was observed in melanomas, commencing with the first clearly recognizable stage of the disease, that of the in situ lesions (Fig 4B). Moreover, there was attenuation in the expression level with advancing stage of melanoma. Whereas these results do not of course discriminate between an initiating or more distal event, decreased expression of MTS1 during tumor progression is obviously not an all-or-none event, but rather a graded process. This suggests a stoichiometric mechanism for carcinogenesis, such as one where a molar deficiency of p16\textsuperscript{INK4} has the net effect of upregulating the activation of CDK4 by cyclin D through loss of competitive inhibition. There would then be more inactivation by phosphorylation of RB1, the target substrate of CDK4, forcing the equilibrium in the direction of unregulated cell growth, thereby fostering malignant transformation. As suggested by our data, the regulatory effects of p16\textsuperscript{INK4} at the late G1 cell cycle checkpoint may be progressively lost as the expression of the gene product attenuates with advancing stage of melanoma. It must be noted, however, that p16\textsuperscript{INK4}-protein and -mRNA levels were normal in some fully evolved melanomas in this series, although abnormal p16\textsuperscript{INK4} function due to intragenic point mutations could not be ruled out. A role for other parallel molecular pathways of oncogenesis is suggested in these cases.

In the report of Reed et al (1995), qualitatively uniform labeling with a polyclonal antibody was observed in 100% of common and atypical nevi and in situ melanomas, but there was reduced labeling of primary invasive and metastatic tumors. The results of Talve et al (1997) using a monoclonal antibody were similar, although some melanomas in situ failed to immunolabel for p16\textsuperscript{INK4}. Taken together with our corroborating data from quantitative immunolabeling with a monoclonal antibody and from in situ RT-PCR with specific primers for p16\textsuperscript{INK4}-mRNA, it can be reasonably concluded that expression of p16\textsuperscript{INK4} is progressively lost with advancement in tumor stage in many cases of melanoma. Moreover, this study and those of both Reed et al (1995) and Talve et al (1997) are concordant for apparently normal expression levels in nevi. In support of the results of Talve et al (1997), however, we observed that mean expression levels are significantly decreased in melanomas in situ. This difference in the results could be methodologic in nature or could reflect variations in the composition of the samples. Neither of the other studies specifically evaluated atypical nevi, which as described herein express normal levels of p16\textsuperscript{INK4} protein and mRNA. Whereas Reed et al (1995) concluded from their data with nevi and melanomas in situ that inactivation of MTS1 is not an initiating event, our observations and those of Talve et al (1997) would be consistent with a more proximal stage for MTS1 inactivation if melanocytic nevi are set aside as formal precursor lesions. Whereas the precise nature of melanocytic nevi and their biologic function, if any, remain largely unknown, a precursor relationship with melanoma has been widely accepted from several lines of evidence (Skender-Kalnenas et al, 1995). If the validity of this conventional model is assumed, such that nevi constitute a proximate morphologic stage in melanoma carcinogenesis (Clark et al, 1984; Elder et al, 1989), then our data showing apparently nominal levels of p16\textsuperscript{INK4}-protein and -mRNA in common and atypical nevi would indicate that loss of its expression is not an initiating event. The alternative interpretation is that nevi may not be a first step in malignant transformation, which could accommodate an early, rate limiting role for MTS1 in the process. There in fact exist empiric observations inconsistent with the position of melanocytic nevi as proximate precursor lesions to melanoma. Among these are the generally low expression levels of proliferation markers in nevus cells (Saenz-Santamaria et al, 1994), the upregulation of apoptocytic factors in nevi (Kankan et al, 1995), and the difficulty in culturing the cells. Such observations, taken together with those reported here, invoke the question of the pathophysiologic role of nevi. One reasonable view is that the spatial association of nevi with melanomas reflects pleiotropic, and divergent, processes sharing a common causation, rather than sequential phases in a continuous, linear progression. This hypothesis, which would account for many of the empiric data, suggests a biologic function for nevi as an adaptive or protective response to melanocytic injury.

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