The Transcriptional Repressor of p16/Ink4a, Id1, Is Up-Regulated in Early Melanomas

David Polsky, Alison Zuyung Young, Klaus J. Busam, and Rhoda M. Alani

Abstract

The helix-loop-helix transcription factor Id1 coordinates cell growth and differentiation pathways within mammalian cells and has been implicated in regulating G1-S phase cell cycle transitions. Recently Id1 has been shown to repress Ets- and E-protein-mediated transactivation of p16/Ink4a. Because the p16/Ink4a protein has been demonstrated to be inactivated in subsets of familial and sporadic melanomas, we sought to determine whether Id1 regulation of p16/Ink4a expression might be involved in the development of this human tumor. Here we evaluate 21 melanocytic lesions at various stages of malignant progression from common melanocytic nevi to metastatic melanomas and examine these lesions for Id1 and p16/Ink4a expression. We demonstrate that Id1 expression correlates with loss of p16/Ink4a expression in melanoma in situ; however, more advanced stages of melanoma do not express Id1 except within perivascular regions, despite overall decreased p16/Ink4a expression in these lesions. Microdissected lesions were evaluated for p16/Ink4a sequence, and invasive melanomas that did not express Id1 were found to have sustained inactivating p16/Ink4a mutations. These data suggest a role for Id1 in regulating p16/Ink4a expression in early melanomas and demonstrate that later genetic changes may provide for irreversible loss of p16 expression in advanced stages of this tumor.

Introduction

Basic HLH DNA binding proteins regulate tissue-specific transcription within multiple cell lineages (1). The Id family of HLH proteins does not possess a basic DNA binding domain and inhibits lineage commitment within multiple cell types through sequestration of basic HLH transcription factors (reviewed in Ref. 2). The Id family member Id1 has been implicated in regulating cellular life span, and we and others have reported delayed senescence of primary human keratinocytes that constitutively express Id1 (3, 4). Because recent studies have illustrated the importance of the pRB tumor suppressor pathway and telomerase activity in regulating primary mammalian cell growth and senescence (5–7), it has been postulated that Id1 regulation of cellular growth and senescence may function through direct regulation of these pathways. Previous studies have demonstrated Id1 reactivation of DNA synthesis in senescent human fibroblasts in cooperation with a mutant SV40 T antigen that is unable to bind pRB, which suggests that Id1 can antagonize the functions of pRb or other members of this tumor-suppressor pathway, including p16/Ink4a (8). More recently, Id1 has been demonstrated to oppose Ets-mediated activation of p16/Ink4a via Ras-Raf-MEK signaling (9), and Id1-null mouse embryo fibroblasts have been shown to senesce prematurely because of increased expression of p16/Ink4a (10).

The p16/pRB pathway has been shown to be deregulated in a large majority of human tumors, either through loss of p16 or pRb function or through deregulated expression of cyclin D or cdk4 (reviewed in Ref. 11). Several mechanisms of inactivation of the p16/pRB pathway have been noted in various tumors and transformed cell lines. Among the most common ways in which these gene products are dysregulated in tumors is through gene deletion; inactivating mutations; epigenetic changes such as promoter methylation, protein sequestration, and inactivation (e.g., viral oncoproteins); and posttranslational modifications (e.g., inactivating phosphorylation events; reviewed in Ref. 12). To date, little is known about the direct transcriptional regulation of genes within the p16/pRb pathway and their role in the development of human malignancies. Here we investigate whether the HLH protein Id1 may play a role in regulating p16/Ink4a expression during the development of melanocytic tumors. We demonstrate that Id1 expression is elevated within the in situ component of malignant melanomas but is not elevated significantly in invasive lesions. Furthermore, we demonstrate that p16/Ink4a expression progressively decreases in association with advancing stages of melanomas and that these invasive lesions have frequently sustained mutations of p16/Ink4a.

Materials and Methods

Immunohistochemistry. Sections (6–7 µm) were blocked with 10% normal horse serum (Vector) in PBS-TX and incubated with monoclonal anti-p16 (Santa Cruz Biotechnology) antibody diluted 1:250 or with polyvalent anti-p19 (AE40; gift of N. Sharpless/R. DePinho, Dana Farber Cancer Institute, Boston, MA) antibody diluted 1:200 in PBS-TX with 1% normal horse serum. Sections were then incubated with the biotinylated horse antiserum secondary antibody (Vector) diluted 1:200 in PBS with 1.5% normal horse serum. The Vectastain Elite ABC kit (Vector) was used for horseradish peroxidase staining of sections.

In Situ Hybridization. Sections (6–7 µm) were processed for in situ hybridization with [α-35S]UTP-labeled cRNA probes, as described previously (13). The Id1 probe template was generated by Y. Jen of Memorial Sloan-Kettering Cancer Center, New York, NY (14).

p16/Ink4a Gene Analysis of Primary Melanomas. Five-µm sections were placed on uncharged slides, stained with H&E, and allowed to air dry. Tumor cells were separated from normal cells by LCM using an Arcturus Pix Cell instrument. Isolated tumor cells were digested overnight with proteinase K at 37°C according to the NIH protocol. DNA was amplified using Qiagen HotStar Taq, 55 cycles, in a multiplex PCR reaction containing primers for both an internal control gene (either a portion of the GAPDH gene or exon 4 or exon 5 of the p53 gene) and exons 1 or 2 of the p16 gene, as described previously (15). Amplified bands were gel purified, and both forward and reverse sequences were obtained in the Sloan-Kettering DNA sequencing facility. Deletions were determined if the internal control band amplified, but the p16 band did not. All of the reactions were performed a minimum of three times.
Id1 IN REGULATING p16/Ink4a EXPRESSION IN EARLY MELANOMAS

Id1 and p16/Ink4a Expression within Melanocytic Lesions. The p16/Ink4a gene has been demonstrated to be inactivated in familial and sporadic melanomas by a variety of mechanisms (16). To determine whether Id1 may play a role in p16/Ink4a regulation during melanomagenesis, we evaluated Id1 and p16 expression in melanocytic lesions at various stages of melanoma progression. Archival samples of five common melanocytic nevi, five atypical (dysplastic) nevi, six primary cutaneous melanomas of different thickness and level of invasion, and five metastatic melanomas were evaluated by immunohistochemistry (p16) and in situ hybridization for Id1 expression (17, 18). We noted decreasing p16 expression as a function of increasing malignant potential in all melanocytic lesions, with 70–90% p16 positivity in compound and dysplastic nevi (Fig. 1, A-2 and B-2), 30–50% positivity in in situ and invasive melanomas (Fig. 1C-3), and <10% positivity in metastatic lesions (Fig. 1D-3). In contrast, in situ evaluation of Id1 expression in these lesions demonstrated no Id1 expression in either compound or dysplastic nevi (Fig. 1, A-2 and B-2) but marked Id1 expression in the epidermis in both the intraepidermal (in situ) melanoma component and neighboring keratinocytes (Fig. 1C-2); however, the invasive components of these melanomas failed to express significantly elevated levels of Id1. Metastatic melanomas demonstrated significantly elevated Id1 expression only around the vasculature within the lesions as has previously been noted in other malignancies (13), which suggests that Id1 may play an important role in early melanomagenesis but is not likely to be necessary for invasion or metastasis of this tumor.

Sequence Analysis of the p16/Ink4a Gene in Primary Melanomas. To determine whether the invasive melanomas that failed to express Id1 possessed a wild-type p16/Ink4a gene, we isolated invasive melanoma cells from the tissues evaluated using LCM. The p16/Ink4a gene was amplified by PCR and sequenced using previously described primers for both an internal control gene (either a portion of the GAPDH gene or exon 4 of the p53 gene) and exons 1 or 2 of the p16/Ink4a gene. Amplified bands were gel purified, and both forward and reverse sequences were obtained. Deletions were determined if the internal control band amplified, but the p16 band did not.

Table 1. Sequence analysis of p16/Ink4a in melanoma samples

<table>
<thead>
<tr>
<th>Tumor depth (mm)</th>
<th>Exon 1</th>
<th>Exon 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Wild type</td>
<td>His337Trp&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>Deleted</td>
<td>Wild-type&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.6</td>
<td>Deleted</td>
<td>Wild-type&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.0</td>
<td>Indeterminate&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Wild-type&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.25</td>
<td>Indeterminate&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Wild-type&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Melanoma in situ</th>
<th>Exon 1</th>
<th>Exon 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not done</td>
<td>Not done</td>
<td>Not done</td>
</tr>
</tbody>
</table>

<sup>a</sup> This mutation has been previously described in a melanoma cell line, but not in a melanoma tissue specimen.

<sup>b</sup> Partial sequence.

<sup>c</sup> Indeterminate, unable to amplify the fragment as well as the internal control.

Fig. 1. Id1 and p16 expression patterns in progressive stages of malignant melanoma development. H&E staining (row 1), in situ hybridization for Id1 expression (row 2), and immunohistochemical (IHC) staining for p16/Ink4a (row 3) in a normal compound nevus (column A), a dysplastic nevus (column B), a primary cutaneous invasive melanoma (column C), and a metastatic melanoma (column D). In lesions with both epidermal and dermal components present: 226, the epidermis; X, the dermis. +, the invasive melanoma component of the lesion in column C. Brown stain on IHC is positive for p16, blue stain is counterstain. Staining patterns are representative of all tissue samples examined. Detailed methods will be provided on request.
ously published primers (15). An internal control gene was simultaneously amplified to assess deletions of p16. Sequence analysis of the p16/Ink4a gene revealed inactivating deletions or missense mutations in invasive melanomas of Breslow level 1.6 mm or greater which suggested a high likelihood of genetic alteration leading to altered p16/Ink4a expression in invasive lesions that failed to express high levels of Id1 (Fig. 2; Table 1).

Discussion

Inherited mutations of the p16/Ink4a gene or the associated cdk4 cyclin-dependent-kinase confer a predisposition to the development of melanoma (19). In sporadic melanomas, however, the frequencies of loss of heterozygosity at the p16/Ink4a locus, p16/Ink4a intragenic mutations, and promoter methylation are extremely low in thin lesions (<10% in lesions of <4 mm), but loss of p16 expression is associated with progression of disease (17, 20, 21), which suggests that other mechanisms allow for decreased p16 expression in early melanomas. Because the transcriptional regulatory protein Id1 has recently been identified as a repressor of p16/Ink4a transcription (9, 10, 22), we sought to determine whether transcriptional inactivation of p16/Ink4a by Id1 might play a role in the initiation or progression of sporadic melanomas.

In the present study, we examined the expression patterns of the HLH protein Id1 and the cell cycle regulatory protein p16/Ink4a in a spectrum of melanocytic lesions from benign nevi to metastatic melanomas. As expected, we noted decreasing expression of the p16/Ink4a gene with malignant progression of melanocytic lesions; however, Id1 expression was limited to the earliest stages of this tumor, suggesting that this early event plays a role in the initiation or progression of sporadic melanomas.

In conclusion, our data provide preliminary evidence for a role for Id1 in functional inactivation of p16/Ink4a in early melanomas. Because these studies suggest that Id1 expression in melanomas is limited to the earliest stages of this tumor, marker studies for Id1 in melanocytic lesions of uncertain malignant potential may play a critical role in the histopathological classification of melanocytic lesions in the future and may help determine the appropriate treatment and prognosis of melanocytic lesions. Although larger studies will need to be undertaken to confirm these findings, they provide a useful paradigm for understanding the mechanisms of development of non-familial melanomas and the potential role for Id1 regulation of p16/Ink4a in the evolution of these tumors.

Acknowledgments

We thank Ronald DePinho and Ned Sharpless for generously providing reagents, and Carole Hazan for technical assistance with melanoma microsections and p16 sequence analysis. We are also grateful to Robert Benezra, Steve Baylin, Bert Vogelstein, and Ken Kinzler for their critical review of the manuscript and helpful discussions.

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*Cancer Res* 2001;61:6008-6011.

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