Down-Regulation of the Novel Gene Melastatin Correlates with Potential for Melanoma Metastasis

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ABSTRACT

We have used differential cDNA display to search for genes whose expression correlates with an aggressive phenotype in variants of the B16 murine melanoma line, B16-F1 and B16-F10. This analysis identified a novel gene, termed melastatin, that is expressed at high levels in poorly metastatic variants of B16 melanoma and at much reduced levels in highly metastatic B16 variants. Melastatin was also found to be differentially expressed in tissue sections of human melanocytic neoplasms. Benign nevi express high levels of melastatin, whereas primary melanomas showed variable melastatin expression. Melastatin transcripts were not detected in melanoma metastases. Within the set of human primary cutaneous melanomas examined, melastatin expression appeared to correlate inversely with tumor thickness. The expression pattern observed suggests that loss of melastatin expression is an indicator of melanoma aggressiveness.

INTRODUCTION

The estimated lifetime risk for developing melanoma in the United States has increased from 1 in 250 in 1980 to more than 1 in 90 in 1996 (1, 2). In addition, melanoma accounted for ~2% of all cancer-related deaths in the United States in 1996 (3). Surgical excision of localized primary cutaneous melanoma may lead to cure in some patients; the overall 5-year survival rate for these patients is ~85% (4). However, there is no cure for patients who present with melanoma metastases to distant sites; their median survival is only 4–8 months (5). One of the most critical issues in the management of patients diagnosed with localized melanoma is identifying those patients who are at highest risk for developing metastases. Tumor thickness remains the primary prognostic factor used in making clinical management decisions. The survival rate for melanoma patients without metastases at presentation decreases with increased tumor thickness. The 8-year survival rate for patients with tumors less than 0.76 mm thick is ~93%, whereas the 8-year survival rate for patients with tumors >3.6 mm thick is less than 35% (6). The identification of a gene that is differentially regulated when melanoma cells attain metastatic potential may lead to increased reliability in determining those patients who have a high risk of developing metastasis.

The B16 series of murine melanoma cell lines is a well-characterized model system for the analysis of tumor progression. Variants of the B16 cell line differ in their ability to form pulmonary metastases after i.v. injection into syngeneic host animals (7, 8). We used differential cDNA display to compare the repertoires of genes expressed by the poorly metastatic B16-F1 cell line and the highly metastatic B16-F10 cell line, with the expectation that some of these differentially expressed genes may function in tumor progression or act as molecular markers of clinical outcome. Using this approach, we identified a novel gene, termed melastatin, that is down-regulated with melanoma tumor progression. Moreover, melastatin expression appears to be inversely related to melanoma thickness, which is presently the best predictor of patient outcome.

MATERIALS AND METHODS

Cell Lines. The B16-F1, B16-F10, B16-F10R, and B16BL-6 cell lines were obtained from the American Type Culture Collection. All cells were grown in DMEM or RPMI supplemented with 10% FBS and antibiotics. Cultures were maintained at 37°C in a 5% CO2-humidified incubator.

Differential cDNA Display. Differential display was performed essentially as described (9). Briefly, total RNA was harvested from B16-F1 cells, and B16-F10 cells were cultured as described using RNAzol according to the manufacturer’s protocol. For each RNA sample, duplicate reverse transcription reactions were carried out in parallel using the oligonucleotide 5’-TTTTTTTTTTTTTTTTCC-3’ as primer. The resulting single-stranded cDNA molecules were then amplified by the PCR. Specifically, reaction mixes containing all four deoxynucleotide triphosphates, arbitrary oligonucleotide 10 mers, the oligonucleotide 5’-TTTTTTTTTTTTTTCC-3’, 10X PCR buffer (Perkin-Elmer), AmpliTaq polymerase (5 units/ml, Perkin-Elmer), 32P-labeled dATP (12.5 mCi/ml; Amersharn), and each of the single-stranded cDNAs described were assembled in 76-well plates. The reactions were then amplified in a Perkin-Elmer 9600 thermal cycler under the following conditions: 94°C for 2 min followed by 40 cycles of 94°C for 15 s, 60°C for 2 min, and 72°C for 30 s. Following the amplification reaction, 15 μl of loading dye containing 80% formamide, 10 mm EDTA, 1 mg/ml xylene cyanole, and 1 mg/ml bromphenol blue were added to each sample and mixed. The samples were fractionated by electrophoresis through an acrylamide-urea gel at ~80 V until the top dye front was about 1 inch from the bottom of the gel. The gel was transferred to 3MM paper (Whatman Paper, Maidstone, United Kingdom) and dried under vacuum. Bands were visualized by autoradiography. PCR bands that were determined to be of interest were excised from the gel, using the autoradiograph as a template guide. The DNA was then extracted from the excised piece of dried gel and used as template for a PCR using the excised cDNA described above. The reamplified DNA was subcloned in pCR II (Invitrogen) and sequenced using an ABI 373 sequencer.

Molecular Cloning. A 187-bp cDNA fragment identified and isolated by differential cDNA display was used to screen a mouse melanocyte cDNA library (10). For the initial screening, ~106 recombinant plaques were screened with the 32P-labeled cDNA fragment at 42°C in 50% formamide, 5X SSC, 0.1% Na2PO4, 50 mm NaPO4 (pH 6.5), 5X Denhardt's solution, and 50 mg/ml sheared denatured salmon sperm DNA for 18 h. Nitrocellulose filters were rinsed at room temperature in 2X SSC and washed at 65°C in 0.1X SSC and 0.1% SDS. Following plaque purification, cDNA inserts were liberated by digestion with EcoRI and subcloned into pBluescript vectors (Stratagene, La Jolla, CA). The cDNA inserts were characterized by standard restriction mapping and sequenced using an ABI 373 automated sequencer.

Northern blot Analysis. Total RNA was isolated for the B16 cell lines using RNAzol following the manufacturer’s protocols. Cell line RNAs (15 μg) were fractionated by electrophoresis through a formaldehyde-agarose (1%) gel, transferred to a Hybond nylon filter (Amersharn), and hybridized with a melastatin cDNA probe. Radiolabeled DNA probes were synthesized using the Klenow fragment of DNA polymerase I with random hexadeoxynucleotides (Boehringer Mannheim) by the method of Feinberg and Vogelstein (11). Conditions for hybridization analysis were as described previously (12). Blots

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Fig. 1. Nucleotide and deduced amino acid sequence of mouse melastatin cDNA, Accession No. AF047714. The mouse melastatin nucleotide sequence is shown above the deduced amino acid sequence. The 187-bp region corresponding to the initial differential display cDNA fragment is underlined.

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were washed to a final stringency of 0.1× SSC, 0.1% SDS at 65°C, and exposed to Kodak XRP film at −80°C with an intensifying screen.

Reverse Transcription-coupled PCR. Total RNA was isolated from a panel of tissue obtained from 8-week-old female C57Bl/6 mice. First-strand cDNA was generated using mouse tissue RNA as template, random hexadeoxyribonucleotides primers, and Superscript II (Life Technologies, Inc., Gaithersburg, MD). Aliquots, corresponding to 200 ng of total RNA, of the first-strand cDNA was subjected to the PCR using two oligonucleotides based on the sequence of mouse melastatin as primers (oligo 1, 5'-GAGGCTGAAAGGAGCTAGAGGT-3'; oligo 2, 5'-CTGGTGCTCCTCTCTCTTGGTG-3'). Control PCR reactions used two oligonucleotide primers for the mouse β-actin sequence (oligo 1, 5'-CTTGGTGCTCCTCTCTCTTGGTG-3'; oligo 2, 5'-ACCCTGGTGCTCCTCTCTCTTGGTG-3'). Reaction products were fractionated by electrophoresis through 1% agarose gel and visualized with UV light, and photographed using a CCD camera.

In Situ Hybridization Analysis. Four-μm sections of formalin-fixed, paraffin-embedded human melanocytic proliferations were deparaffinized, rehydrated, and postfixed with 4% paraformaldehyde/PBS for 15 min. After washing with PBS, sections were digested with 2 μg/ml proteinase K at 37°C for 15 min and again incubated with 4% paraformaldehyde/PBS for 10 min. The tissue sections were then washed with PBS, incubated with 0.2 N HCl for 10 min, washed, and dehydrated with 70% ethanol and 100% ethanol. The sections were then incubated with 2 × SSC, 50% formamide/PBS at 37°C for 15 min and once again incubated with 4% paraformaldehyde/PBS for 10 min. The sections were treated with 50% acetic anhydride/1 M triethanolamine for 10 min, washed, and dehydrated with 70% ethanol and 100% ethanol. Hybridizations were performed with 32P-radiolabeled (5 × 10^6 cpm/ml) cRNA probes encoding a 1.1-kb segment of the coding region of the human melastatin cDNA (clone fohy030) and a 1-kb segment of the coding region of the human H4 histone gene in the presence of 50% formamide, 10% dextran sulfate, 1× Denhardt’s solution, 600 mM NaCl, 10 mM DTT, 0.25% SDS, and 10% dextran sulfate. After autoradiography with the melastatin probe, the filter was stripped and reprobed with a radiolabeled β-actin cDNA fragment. Note the excess of B16-F1 RNA on this filter.

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Identification of Melastatin. We compared the repertoires of genes expressed by the poorly metastatic mouse melanoma cell line B16-F1 and the highly metastatic variant B16-F10 in the hopes of identifying genes whose expression correlates with metastatic potential. Sets of reactions, corresponding to 1000 pairs of oligonucleotide primers, were run against these two cell lines. This analysis lead to the isolation and sequencing of 98 differential display “bands.” Only 1 of the 98 bands corresponded to a gene that exhibited properties, i.e., differential expression in both the B16 cell lines and in clinical samples of human melanoma, that warranted further investigation and description in this report.

A differential display analysis using the arbitrary oligonucleotide primer (5'-GTGACATGCC-3') and the oligonucleotide (5'-TTTTTTTTTTTC3') identified a cDNA fragment that appeared to be more intense in the B16-F1 lane relative to the B16-F10 lane (data not shown). Sequence analysis of the cDNA fragment revealed it to be 187-bp in length with no similarity to any of the sequences in the nucleotide database. To isolate a full-length cDNA, a cDNA library made from normal mouse melanocytes was screened using a probe made from the 187-bp cDNA fragment. The primary screening of 10^6 plaque-forming units of the cDNA library yielded three positive clones. Restriction mapping and partial sequencing of the three cDNA clones showed that they are independent, overlapping clones of similar size. Sequence analysis revealed the largest clone to be 2722 bp in length with the 187-bp cDNA fragment corresponding to bp 1726–1913 (Fig. 1). The cDNA had a single large open reading frame of 1626 bp, predicted to encode a protein of 542 amino acids in length. Searches of the sequence databases using the cDNA and predicted amino acid sequences revealed no significant similarities to any proteins of known function. Hydropathy analysis of the deduced polypeptide shows neither a signal sequence nor identifiable membrane-spanning domains. Based on further observations described in this report, we termed this novel gene melastatin.

To isolate a human melastatin cDNA, we screened a human eye cDNA library using the mouse melastatin cDNA as a probe. A partial human cDNA clone was isolated that corresponds to nucleotides 42–1821 of mouse melastatin. A comparison of mouse and human melastatin sequences showed a high degree of sequence similarity between these clones, specifically, 85% identical at the nucleotide level and 94% identical at the amino acid level.

**Melastatin Expression in Mouse Melanoma Cell Lines and Tissues.** To investigate the expression of melastatin, we used a mouse melastatin cDNA fragment to probe Northern blots containing total RNA from the four B16 variants: B16-F1, B16-F10, B16-F10Lrfi, and B16BL-6. These four cell lines differ in their ability to form pulmonary metastases after injection into the tail veins of syngeneic hosts. The B16-F1 and B16-F10Lrfi cell lines are poorly metastatic and form few lung colonies (7, 8, 13, 14). The B16-F10 cell line, in contrast, is highly metastatic, forming numerous pulmonary metastases after injection into syngeneic hosts (7, 8). The B16BL-6 cell line forms a quantity of pulmonary metastases intermediate between the numbers caused by B16-F1 and B16-F10 cell lines (15). As seen in Fig. 2, a probe for melastatin detected an ~2.8-kb transcript in all four of the B16 cell lines. This transcript is expressed at high levels in B16-F1 and B16-F10Lrfi cells, at intermediate levels in B16BL-6, and at low levels in B16-F10. The differential expression of melastatin by
Fig. 5. *In situ* hybridization analysis of melastatin expression in human melanocytic proliferations. A. dermal nevus. *Panel 1*: nests of banal nevomelanocytes are present in the superficial dermis (bright-field, melastatin antisense probe). *Panel 2*: abundant melastatin mRNA is revealed with dark-field examination as bright white dots localized to the dermal nests of nevomelanocytes (dark field, melastatin antisense probe). B. superficial spreading melanoma, radial growth phase. In *panel 1*, this radial growth phase melanoma is predominantly intraepidermal and has an associated host inflammatory response (bright field, melastatin antisense probe). Abundant melastatin mRNA is revealed with bright-field examination as black dots localized to the radial growth phase (1), and in *panel 2* with dark-field examination as bright white dots (dark field, melastatin antisense probe). C. superficial spreading melanoma, radial and vertical growth phase. In *panel 1*, an intraepidermal component and a heterogeneous dermal vertical growth phase are observed with bright-field examination (bright field, melastatin antisense probe). Melastatin mRNA is localized to the intraepidermal component and portions of the dermal component. In *panel 2*, melastatin mRNA is not detected in many of the tumor cells in the vertical growth phase (dark field, melastatin antisense probe). *Panel 3*: histone mRNA was observed in cells scattered throughout the dermal component (data not shown). D. metastatic melanoma. In *panel 1*, dermal melanoma nests are present in this cutaneous metastasis (bright field, melastatin antisense probe). In *panel 2*, melastatin mRNA is not detected (dark field, melastatin antisense probe). *Panel 4*: histone mRNA was present in scattered basal layer keratinocytes and melanoma cells (data not shown).

The tissue distribution of melastatin was examined in a panel of murine tissues using reverse transcription-coupled PCR. Melastatin expression was not detected in RNA derived from total brain, lung, liver, heart, kidney, spleen, and small intestine. Melastatin transcripts were detected only in the eye and the control B16-F1 cell line (Fig. 3).

*Melastatin Expression in Human Melanoma Cell Lines and Tissue Sections of Melanocytic Neoplasms.* Melastatin transcripts were not detected in any melanoma cell lines derived from human melanoma metastases, whereas the 2.8-kb *melastatin* transcript was identified in the B16-F1 cell line control (Fig. 4).

*Melastatin* expression was examined in formalin-fixed tissue sections of 45 human melanocytic neoplasms by *in situ* hybridization (Figs. 5 and 6). *Melastatin* mRNA was present throughout all 20 benign melanocytic nevi studied and was observed only in cells with melanocytic differentiation (Fig. 5a). Six of these 20 nevi were dysplastic melanocytic nevi with marked cytological atypia. On the other hand, *melastatin* mRNA was diffusely expressed in 14 of 20 primary cutaneous melanomas, ranging in thickness from 0 mm (*in situ* melanoma) to 2.95 mm (Fig. 5b). Focal loss of *melastatin* mRNA expression by the dermal component of the tumor was noted in 6 of 20 primary cutaneous melanomas, ranging in thickness from 0.5 to 6.0 mm (Fig. 5c). Notably, loss of *melastatin* expression appears to
DISCUSSION

Melastatin is a novel, differentially expressed gene that was identified by differential display analysis of B16-F1 and B16-F10 murine melanoma cell lines. The expression of melastatin in four independent variants of the B16 melanoma showed an inverse correlation with the reported ability of these lines to form lung colonies after injection into the tail veins of syngenic animals. The inverse correlation between melastatin expression and the progression of melanomas was also seen in human melanoma cell lines derived from metastatic melanomas, melastatin mRNA was not observed in any of five cases of metastatic melanoma (Fig. 5d). The control riboprobe for H4 histone mRNA was positive in all cases. H4 histone RNA-bearing cells were observed in areas of the melanomas where melastatin mRNA was not detected, serving as a positive control for tissue mRNA preservation (data not shown).

correlate with the thickness of the primary cutaneous melanomas examined (Fig. 7). In contrast to benign melanocytic nevi and primary cutaneous melanomas, melastatin mRNA was not observed in any of five cases of metastatic melanoma (Fig. 5d). The control riboprobe for H4 histone mRNA was positive in all cases. H4 histone RNA-bearing cells were observed in areas of the melanomas where melastatin mRNA was not detected, serving as a positive control for tissue mRNA preservation (data not shown).

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Fig. 6. Summary of the in situ hybridization analysis of melastatin in biopsies of 45 human melanocytic proliferations. Each symbol represents a different tumor. Loss of melastatin mRNA ranged from 10% to >75% of tumor cells.

metastatic potential of these transfected cell lines to untransfected or mock-transfected controls.

The expression of melastatin appears to be restricted to normal melanocytes, melanocytic proliferations, and some melanomas; its expression in the pigmented choroid of the eye remains to be examined. A functional role for melastatin in the normal melanocyte is as yet unknown. Our data does not support a role for melastatin in the pigmentation of melanomas. Many of the metastatic lesions and locally invasive melanomas examined by in situ hybridization were highly pigmented, despite the loss of melastatin expression (data not shown). Another intriguing possibility is that melastatin functions in the differentiation of melanocytes from their neural crest-derived precursors, which are migratory in nature (17). In this scenario, melastatin may function as part of a signal to stop the migration of the neural crest-derived precursors. This premise could be explored by examining the expression of melastatin during embryogenesis to determine whether it is synchronous with cessation of migration.

We have shown the loss of melastatin expression to be correlated with progression to a metastatic phenotype in melanomas. Recently, a number of additional novel genes have been identified that are thought to function in the development of metastases (18–20). Specifically, a number of markers have been identified that are associated with melanocytic tumor progression (21–24), including genes identified using the differential display technique (25, 26). To date, however, none of these genes or proteins have shown sufficient correlation with human primary melanoma thickness and metastasis to encourage large studies evaluating them as potential prognostic indicators. Despite extensive research studying immunohistochemical and molecular markers of tumor progression, tumor thickness remains the most reliable indicator of metastatic potential. Unfortunately, melanoma thickness is not always an accurate prognostic indicator; patients with very thin tumors may die of melanoma, and patients with thick melanomas may experience long-term disease-free survival. Identification of a prognostic indicator that is a useful adjunct to tumor thickness may allow for more accurate determination of those patients with early stage melanoma who will develop metastatic disease.

Heightened awareness has led to an increase in the diagnosis of early stage cutaneous melanoma in recent years (1). In those patients...
with localized melanoma at the time of diagnosis, the measurement of tumor thickness remains the gold standard for determining treatment. Patients with very thin tumors can be successfully treated with excision of the primary melanoma. However, despite the publication of a number of elegant models for determining melanoma prognosis (6, 27–29), patients with melanomas of intermediate thickness present the greatest prognostic challenge. Some of these patients will be cured with surgical excision, whereas others will die of metastatic melanoma despite complete excision of the primary tumor. The finding that melastatin mRNA expression is variably down-regulated in melanomas of intermediate thickness is of interest. Further studies into the relationship of melastatin expression to metastasis and outcome in patients with localized melanoma may show that melastatin is a reliable indicator of metastatic potential independent of tumor thickness. In addition, investigations into the functional characteristics of melastatin may allow for the development of novel melanoma treatment strategies for patients with this often devastating disease.

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