Mitogen-activated Protein Kinase Activation Is an Early Event in Melanoma Progression

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ABSTRACT

Purpose: Melanoma is the most common cause of death from cutaneous malignancy, and is the cancer that is most rapidly rising in incidence. Because current therapeutic methods for metastatic melanoma are poorly efficacious, enhanced understanding of signal transduction in melanoma progression is warranted. Prior experimental studies in murine models and human tissues have shown a correlation among activation of mitogen activated protein kinase (MAPK) signaling, angiogenesis, and tumorigenesis. Because of these findings, we wanted to assess the role of MAPK signaling in melanoma progression and angiogenesis.

Experimental Design: We studied expression of phosphorylated (active) MAPK and two target genes known to be induced by MAPK signaling, tissue factor and vascular endothelial growth factor, in 131 melanocytic lesions, ranging from atypical nevi to metastatic melanoma.

Results: We observed little staining for activated (phosphorylated) MAPK and low amounts of angiogenesis in atypical nevi, but angiogenesis and MAPK activation were activated in radial growth melanoma and in later stage lesions.

Conclusions: Our findings implicate MAPK activation as an early event in melanoma progression, and MAPK may be a potential target for pharmacologic intervention.

INTRODUCTION

Melanoma is a common and lethal form of skin cancer. Whereas melanoma ranks as the third most common form of skin cancer, after basal and squamous cell carcinoma, melanoma causes far more deaths because of recurrences and distant metastases (1). In addition, melanoma is highly resistant to chemotherapy and radiation; thus, metastatic disease has a poor prognosis. Because melanoma is a common and poorly treatable malignancy in advanced stages, knowledge of signal transduction pathways in melanoma progression may provide novel targets for melanoma therapeutics and prevention.

Melanoma is a classic example of tumor progression. At least some cutaneous melanomas are thought to arise from precursor lesions termed atypical nevi (2). Patients with germ-line mutations in the tumor suppressor gene p16ink4a have an increased rate of melanoma, suggesting that loss of this tumor suppressor gene is involved in melanoma progression (3); however, the point at which p16ink4a is lost is not clear (4). Clinically the transition from atypical nevus to radial growth melanoma has been observed, as has the transition from radial growth melanoma to vertical growth melanoma. Various mutations have been observed in late-stage melanoma, such as activation of ras or loss of the tumor suppressor gene PTEN (5, 6). However, the alterations in signal transduction, which accompany the transition from atypical nevus to radial growth melanoma are not well understood. In this study, we examined a large series of melanocytic neoplasms for expression of activated P-MAPK (3) and two target genes of MAPK activation, VEGF and TF (7–9). We found that P-MAPK, and its target genes, VEGF and TF, are observed in radial growth melanoma but not in atypical nevi, suggesting that MAPK activation is an early event in melanoma progression.

MATERIALS AND METHODS

We studied formalin-fixed, paraffin-embedded blocks (117 malignant melanoma and 14 nevi) from the archives of the Department of Pathology and Laboratory Medicine at Emory University Hospital, Atlanta, Georgia. Clinical, pathologic, and follow-up information was obtained from surgical pathology reports and the Winship Cancer Center Oncology Data Bank, Emory University School of Medicine, Atlanta, GA. The nevi studied were composed of 3 junctional, 4 minimal atypical, 3 mildly atypical, and 3 moderate-severe atypia.

Immunohistochemistry. Five-μm sections were immunostained for P-MAPK (1/30; New England Biolabs, Beverly, MA), VEGF (1/160; Santa Cruz Biotechnologies, Santa Cruz, CA), TF (1/160; American Diagnostica, Greenwich, CT), and CD31

The abbreviations used are: P-MAPK, phosphorylated mitogen-activated protein kinase; MAPK, mitogen-activated protein kinase; VEGF, vascular endothelial growth factor; TF, tissue factor.
(1/80; Dako Corporation, Santa Barbara, CA) using an avidin-biotin complex method, steam heat-induced epitope retrieval, and the DAKO Autostainer (Dako). An avidin-biotinylated enzyme complex kit (LSAB 2; Dako) was used according to the manufacturer’s specifications with hematoxylin as counterstain. Positive controls were a hemangioma for P-MAPK, myometrial blood vessels (VEGF, CD31, and P-MAPK), and a known TF-positive breast carcinoma. Negative controls had the primary-specific antibody replaced by buffer. Specificity of the P-MAPK antiserum has been demonstrated previously using melanoma protein (10). P-MAPK, VEGF, and TF were quantitated as intensity of immunostain (0–3 +) and percentage of immunoreactive MM/nevi cells (0–100%). CD31 was visually semiquantitated as mean and maximum vessel density by two pathologists (A. Z-P. and C. C.) in two “hot spots” at ×200 magnification, who viewed the slides at the same time but counted them independently, and the MVD was calculated as the average of the measurement of the pathologists (11, 12).

**Table 1** Depth of invasion related to marker expression in 117 malignant melanomas and 14 nevi

<table>
<thead>
<tr>
<th></th>
<th>P-MAPK</th>
<th>VEGF</th>
<th>TF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td><strong>Angiogenesis</strong></td>
<td>CD31</td>
<td>MVD</td>
<td>Mean (std)</td>
</tr>
<tr>
<td>Total lesions</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td><strong>Clark’s Level</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>12</td>
<td>11 (92%)</td>
<td>1 (8%)</td>
</tr>
<tr>
<td>II</td>
<td>29</td>
<td>27 (93%)</td>
<td>2 (7%)</td>
</tr>
<tr>
<td>III</td>
<td>15</td>
<td>12 (80%)</td>
<td>3 (20%)</td>
</tr>
<tr>
<td>IV</td>
<td>49</td>
<td>43 (88%)</td>
<td>6 (12%)</td>
</tr>
<tr>
<td>V</td>
<td>9</td>
<td>5 (56%)</td>
<td>4 (44%)</td>
</tr>
<tr>
<td><strong>Breslow Thickness</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In situ</td>
<td>12</td>
<td>11 (92%)</td>
<td>1 (8%)</td>
</tr>
<tr>
<td>&lt;1 mm</td>
<td>39</td>
<td>35 (90%)</td>
<td>4 (10%)</td>
</tr>
<tr>
<td>1–3 mm</td>
<td>42</td>
<td>33 (79%)</td>
<td>9 (21%)</td>
</tr>
<tr>
<td>&gt;3 mm</td>
<td>22</td>
<td>19 (86%)</td>
<td>3 (14%)</td>
</tr>
<tr>
<td>Nevi</td>
<td>14</td>
<td>3 (21.5%)</td>
<td>11 (78.5%)</td>
</tr>
</tbody>
</table>

Fig. 1 Immunohistochemical and histological analysis of nevi and melanoma. The top row (A–D) represents immunohistochemistry for P-MAPK (A), VEGF (B), CD31 (C), and TF (D) in an atypical nevus. The second row represents immunohistochemistry for P-MAPK (E), VEGF (F), CD31 (G), and TF (H) in a radial growth melanoma. The third row represents immunohistochemistry for P-MAPK (I), VEGF (J), CD31 (K), and TF (L) in a vertical growth melanoma.

**Cell Culture/Western Blot Analysis.** PMWK is a radial growth melanoma cell line characterized previously (13). Primary human melanocytes were obtained from the Emory Skin Disease Research Center Tissue Culture Core and cultured in...
Activation of MAPK in Melanoma

Table 2  Angiogenesis: correlation with malignant melanoma parameters and prognosis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Angiogenesis (CD31 MVD) (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clark’s level (I–II vs. III–V)</td>
<td>0.023</td>
</tr>
<tr>
<td>TF (+ vs. −)</td>
<td>0.062</td>
</tr>
<tr>
<td>Breslow thickness (In situ vs. &lt;1 mm vs. 1–3 mm vs. &gt;3 mm)</td>
<td>0.077</td>
</tr>
<tr>
<td>P-MAPK (+ vs. −)</td>
<td>0.717</td>
</tr>
<tr>
<td>VEGF (+ vs. −)</td>
<td>0.952</td>
</tr>
<tr>
<td>Overall survival</td>
<td>0.636</td>
</tr>
<tr>
<td>Disease-free survival</td>
<td>0.488</td>
</tr>
</tbody>
</table>

Table 3  Relationship of TF and other parameters in malignant melanoma

<table>
<thead>
<tr>
<th>Parameter</th>
<th>TF (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clark’s level (I–II vs. III–V)</td>
<td>0.019</td>
</tr>
<tr>
<td>VEGF (+ vs. −)</td>
<td>0.090</td>
</tr>
<tr>
<td>CD31 MVD</td>
<td>0.062</td>
</tr>
<tr>
<td>P-MAPK (+ vs. −)</td>
<td>0.120</td>
</tr>
<tr>
<td>Breslow thickness (In situ vs. &lt;1 mm vs. 1–3 mm vs. &gt;3 mm)</td>
<td>0.093</td>
</tr>
<tr>
<td>Overall survival</td>
<td>0.528</td>
</tr>
<tr>
<td>Disease-free survival</td>
<td>0.844</td>
</tr>
</tbody>
</table>

RESULTS

The mean age of the 117 patients studied with MM was 60 years (range, 22–92). Sixty-three (54%) were males, 54 (46%) were females. The Clark level and Breslow depth of invasion of the MM studied are detailed in Table 1, relative to P-MAPK, VEGF, and TF expression. The six atypical nevi were negative for activated MAPK expression, but MAPK activation was noted in both radial and vertical growth phases of MM (Fig. 1). Lymph node status was not available in 32 patients. Follow-up at the time of this report revealed 6 cases of local recurrence and 18 cases of distant metastases among the patients in which follow-up could be obtained. Mean follow-up in 96 patients was 60.8 months (range, 1–227). Expression of P-MAPK was not observed in only 21.5% of benign nevi, all of which had mild atypia, and, thus, were not likely to be diagnostically confused with melanoma (Fig. 1).

Table 2 shows that angiogenesis as CD31 mean MVD correlates significantly with the Clark level of the MM studied (P = 0.03) and tended to correlate with TF expression (P = 0.06), but showed no significant relationship to Breslow thickness, P-MAPK, and VEGF expression, or overall and disease-free survival (P = >0.05). P-MAPK tended to correlate with Clark level (P = 0.08), whereas VEGF did not, but neither VEGF nor P-MAPK expression (angiogenic markers) correlated with Breslow thickness, lymph node status, or overall survival.

Table 3 indicates the statistical relationship between TF and clinical, pathologic, and follow-up parameters. TF expression correlates significantly with Clark level (P = 0.019) and VEGF expression (P = 0.003), and tended to correlate with angiogenesis as mean MVD of both the mean and maximum CD31 counts (P = 0.06), but did not correlate with P-MAPK expression. Breslow thickness, or overall and disease-free survival (P = > 0.05). TF expression increased from 28% to 52% to 70% in MM showing VEGF expression of 0–1+, 2+, and 3+ intensity, respectively.

To additionally confirm differences in MAPK signaling between primary melanocytes and radial growth melanoma cells, we performed Western blot analysis comparing primary human melanocytes with radial growth melanoma (PMWK cells). When cultured in basal medium (DMEM supplemented with 5% FCS), primary melanocytes showed low expression of activated MAPK expression compared with constitutive activation of MAPK in radial growth melanoma cells (Fig. 2).

DISCUSSION

The major cause of death from melanoma is because of distant metastases. The major prognostic markers of melanoma, Breslow thickness and Clark levels, are biological measures of tissue invasion (15). Melanoma is characterized by a radial...
growth phase, which proliferates primarily along the dermo-epidermal junction. Radial growth phase melanoma cells accumulate additional mutations, including activation of ras oncoproteins and loss of the PTEN tumor suppressor gene (5, 16–18). Activation of ras in human and murine melanoma confers the ability of cells to invade the dermis in an expandable and proliferative pattern, and produce angiogenic factors such as VEGF (19–22). The ability of melanoma cells to undergo proliferation in three dimensions is clinically known as vertical growth phase melanoma (15, 23). As expected from experimental data, clinical vertical growth phase melanoma is a highly angiogenic and proliferative lesion.

Several genes have been associated with highly aggressive behavior in vertical growth and metastatic melanoma. These genes include αβ integrin and markers thought previously to be endothelial specific, such as VEGF receptors VEGFR1 and VEGFR2, VE cadherin, and ephrins (24–27). This phenomenon has been termed vasculogenic mimicry. Recently, two groups independently isolated rho C through gene chip analysis as a mediator of metastatic behavior (28, 29). Whereas much knowledge has been gained through these approaches, the events that mark the transition from atypical nevus to early melanoma are not well understood. This is attributable in part to the lack of relevant cell lines, especially because atypical nevi are rarely cultured and do not proliferate well in culture (30). Recently, Id1, a protein which down-regulates the tumor suppressor gene p16ink4a, has been shown to be expressed in radial growth melanoma (31). Down-regulation of p16ink4a may allow MAPK-mediated proliferation and escape from senescence (32), as activation of MAPK promotes either senescence or transformation, depending on the status of p16ink4a (33–36).

Thus, several lines of evidence point toward MAPK as a potential mediator of melanocytic tumor progression (23, 35, 36). Recently, mutations in B-raf have been detected in 59% of melanoma cell lines and 80% of short-term cultures of primary melanomas, and the B-raf mutations in these cells have been shown to cause activation of MAPK signaling (37). These studies additionally confirm the central role of MAPK signaling in malignant melanoma. Our study has the advantage of determining the timing of MAPK activation in melanoma tumor progression, and has the advantage that these studies can occur in paraffin sections. Targets of MAPK include the proangiogenic markers VEGF and TF (38, 39). We found that expression of activated MAPK and its targets, VEGF and TF, are observed in radial growth melanoma and later stages, but not in its immediate precursors. In culture, MAPK activation has been observed in proliferating primary melanocytes in the presence of growth-promoting agents, such as phorbol ester, but is decreased on senescence or removal of growth-promoting agents (40). In contrast, radial growth melanoma cells grow readily in vitro in the absence of growth-promoting agents. We observed constitutive expression of activated MAPK is observed in radial growth melanoma cells compared with primary melanocytes.

Decreased expression of activated MAPK has been noted in some specimens in more advanced melanoma. The reasons for this are not currently known, but may include alternative signaling pathways activated in advanced melanoma. Advanced melanomas have been shown previously to express high levels of reactive oxygen species, and we have shown recently that increased reactive oxygen can stimulate both angiogenesis and tumorigenesis in p16-deficient NIH 3T3 cells (41). Cells transformed by the reactive oxygen species generating enzyme nox-1 show relatively low levels of MAPK activation, suggesting that reactive oxygen species may assume some of the role of tumorigenesis from MAPK in more advanced lesions.

Our findings may help explain the conflicting findings between angiogenesis and tumor progression in melanoma. Several studies have implicated a link between prognosis and microvessel density, whereas other studies have not (42–49). Our findings suggest that the angiogenic switch occurs early in melanoma, whereas later events are required for three-dimensional growth and distant metastases. In addition, our studies suggest that pharmacologic inhibition of MAPK signaling may be of benefit in the prevention and treatment of cutaneous melanoma (50).

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