Malignant Transformation of Melanocytes to Melanoma by Constitutive Activation of Mitogen-activated Protein Kinase Kinase (MAPKK) Signaling*

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Malignant melanoma is the cancer with the most rapid increase in incidence in the United States. Ultraviolet light and deficiency of the p16INK4a gene are known factors that predispose one to the development of malignant melanoma. The signal transduction pathways that underlie the progression of melanoma from their precursors, atypical nevi, are not well understood. We examined activation of the MAP kinase pathway in atypical nevi and melanoma cells and found that this pathway is activated in melanomas. To determine the functional significance of this activation, we introduced constitutively active MAP kinase kinase (MAPKK) into immortalized melanocytes. The introduction of this gene into melanocytes leads to tumorigenesis in nude mice, activation of the angiogenic switch, and increased production of the proangiogenic factor, vascular endothelial growth factor (VEGF), and matrix metalloproteinases (MMPs). Activation of MAP kinase signaling may be an important pathway involved in melanoma transformation. Inhibition of MAP kinase signaling may be useful in the prevention and treatment of melanoma.

Malignant melanoma is a major cause of morbidity and mortality. Melanomas often arise from precursor lesions called atypical nevi (1). Although the prognosis of thin melanoma is excellent, prognosis decreases with increased thickness of the lesions. The diminished prognosis is due mainly to the well-established tendency of melanoma to metastasize. In addition, melanomas are highly resistant to most forms of chemotherapy and radiation; therefore, cure of the disseminated disease is uncommon. Thus, there is an urgent need to understand the signal transduction pathways underlying transformation of melanocytes to melanoma.

Several histologic and immunohistochemical markers have been shown to correlate with prognosis. The first and still most widely used markers of melanoma prognosis are Breslow thickness and Clark’s levels (2, 3). Breslow thickness measures the width of the lesion, and lesions >0.75 mm have a distinctly worse prognosis than thinner melanomas. Clark’s levels measure the depth of invasion of the melanoma from the dermo-epidermal junction to deeper layers of the dermis and subcutaneous fat. Both of these markers are measures of the ability of melanoma cells to grow three dimensionally (4, 5). More recently, other genes have been shown to be expressed or repressed in different stages of melanoma progression (6, 7). Telomerase is present in radial and vertical growth melanomas but not in atypical nevi (8, 9). Various integrins have been shown to be expressed in advanced melanomas and correlate with decreased apoptosis and invasion (10, 11). A potent angiogenesis factor, VEGF, is highly expressed in advanced melanoma. Finally, expression of the small G protein rhoC is associated with increased metastatic ability of melanoma (12, 13). However, the signal transduction pathways associated with melanoma transformation are not well understood.

We have previously noted that an inverse correlation exists between the expression of activated MAP kinase and the degree of malignancy in tumors derived from endothelium (4, 14). Other investigators have demonstrated a direct correlation between MAP kinase expression and the degree of malignancy in other tumors (15). These findings suggest that the role of MAP kinase in malignancy may be tissue specific. We examined the expression of activated MAP kinase in human nevi and melanomas and found increasing expression in malignant cells, particularly in early (radial growth) melanoma (16). Furthermore, we demonstrate that MAP kinase activation functionally contributes to the development of melanoma, as the introduction of a constitutively active MAPKK into melanocytes leads to transformation in vivo. The activation of MAP kinase results in the activation of AP-1 but not the activation of NFκB. Inhibition of MAP kinase signaling (17) may provide a therapeutic strategy for the prevention and treatment of melanoma.

MATERIALS AND METHODS

Immunohistochemistry—Paraffin-fixed sections of nevi and melanomas were stained with an antibody specific for phosphorylated MAP kinase according to the procedure of Arbiser et al. (18).

Generation of Cell Lines—L10BIOBR cells are an immortalized murine melanocyte cell line that proliferates best in the presence of exogenous phorbol ester (G. P. Dotto, Cutaneous Biology Research Center, Harvard Medical School, Charlestown, MA) (19). These cells were maintained in F-10 nutrient mixture (Ham’s medium) with 7% horse serum. L10BIOBR cells were infected with retroviruses encoding green fluorescent protein (pDIVA-GFP) (A. Kowalczyk, Emory University) or a

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1 The abbreviations used are: VEGF, vascular endothelial growth factor; MAP, mitogen-activated protein; MAPK, MAP kinase; MAPKK, MAPKK kinase; AP-1, activator protein 1; NFκB, nuclear factor κB; TIMP, tissue inhibitor of matrix metalloproteinase; GFP, green fluorescent protein; RT, reverse transcription; MMP, matrix metalloproteinase.

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constitutively active MAPKK mutant, LIDA/MANA (C. Marshall, Institute of Cancer Research, London). Both vectors encode puromycin resistance, and cells were selected in 2 μg/ml puromycin and pooled (20). GFP expression was confirmed by fluorescence microscopy, and expression of the active MAP kinase kinase gene was confirmed by performing Western blot analysis with an antibody specific to phosphorylated MAP kinase. Protein extracts were prepared as described by Arbiser and co-workers (21). Expression of the constitutively active MAPKK was confirmed using antibody 177 to rabbit MAPKK (C. Marshall, London).

**Western Blotting** — Cells were lysed in lysis buffer containing 20 mM Tris HCl (pH 7.5), 150 mM NaCl, 1% (v/v) Triton X-100, 10% glycerol, 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM benzamidine, 1 mM phenylmethylsulfonylfluoride, and 1 mM Na3VO4. Protein concentration was determined by the Bradford assay using bovine serum albumin as a standard. Samples were treated with Laemmli sample buffer and heated to 90 °C for 5 min prior to SDS-PAGE (National Diagnostics, Atlanta, GA) and transfer to nitrocellulose membranes. The membranes were then blocked with 5% nonfat dry milk in Tris-buffered saline with Tween 20 and subsequently incubated with the appropriate antibody for immunoblotting. The anti-Erk2 monoclonal antibody was from Santa Cruz Biotechnology, and anti-phospho-MAPK, phospho-c-Jun (Ser73), phospho S6 ribosomal protein (Ser235-Ser236), phospho S6 ribosomal protein (Ser240-Ser244), and phospho p70 S6 kinase (Thr421-Ser424) antibodies were from Cell Signaling Technologies (Beverly, MA).

**Assays of Matrix Metalloproteinase Bioactivity** — Cells were grown to ~75% confluence in Dulbecco’s Modified Eagle Medium supplemented with 10% fetal calf serum. After washing with phosphate-buffered saline, the medium was replaced with Cellgro serum-free medium (Mediatech, Herndon, VA). Substrate gel electrophoresis (zymography) was conducted as described by us previously (14). Briefly, Type I gelatin was added at a concentration of 1 mg/ml to the standard Laemmli acrylamide polymerization mixture (22). Conditioned media from equivalent numbers of cells were diluted 3:1 with sample buffer (10% SDS, 4% sucrose, 0.25 M Tris, pH 6.8, and 0.1% bromphenol blue) and electrophoresed as described previously. At the end of electrophoresis, gels were rinsed in 2.5% Triton X-100 for 30 min and then incubated overnight in substrate buffer (50 mM Tris, pH 8.2, 5 mM CaCl2, 0.02% NaN3). The gels were stained with 0.5% Coomassie Blue R-250 in acetic acid/isopropyl alcohol/H2O (1:3:6) and destained in the same buffer in which the Coomassie Blue was prepared. The densitometry of destained areas was quantified using a Datascopy GS Plus scanner connected to Macintosh II computer with Macimage software (Xerox Imaging Systems).

**Assay of Tissue Inhibitor of Matrix Metalloproteinase Bioactivity** — TIMP bioactivity was quantified using conditioned media from equal numbers of L10BIOBR cells expressing active MAPKK or GFP vector

**Fig. 1. Immunohistochemical analysis of nevi and melanoma.** The top panel represents immunohistochemistry for phospho MAPK on an atypical nevus, and the bottom panel represents an invasive melanoma (40×).

**Fig. 2. A.** Analysis of phospho MAP kinase and total MAP kinase expression in immortalized (L10BIOBR-GFP) (−) and transformed melanocytes (L10BIOBR-MAPKK) (+). The bands at the top represent an immunoblot with an antibody specific for phosphorylated MAPK, the gel in the middle represents total MAPKK protein showing overexpression of the retroviral transgene, and the gel at the bottom represents an immunoblot with an antibody for total MAPK. **B.** Introduction of MAPKK into L10BIOBR cells induces a transformed morphology. The left panel shows vector control (GFP)-transduced melanocytes, whereas the right panel shows MAPKK-expressing melanocytes.
control. A solid collagen film assay was performed using C14-labeled collagen as described by us previously (14).

In Vivo Tumorigenesis—One million cells were injected subcutaneously into 4–5 week old male nude mice (Charles River) in the presence of a small quantity of trypan blue to mark the inoculation site. Tumors were excised after one month, fixed in formalin, and subjected to histologic and in situ hybridization. Tumor volume was calculated according to the formula \( w^3 \times 0.52 \), where \( w \) represents the shortest dimension (14).

In Situ Hybridization for Histone H3—In situ hybridization was performed on 4 mm-thick sections of formalin-fixed, paraffin-embedded tissue. Details of in situ hybridization have been reported previously (18, 23, 24). The emulsion was developed with hematoxylin (23, 25).

RT-PCR for VEGF—Total RNA was isolated using TRI Reagent (Sigma). RT-PCR was done with Promega Accession RT-PCR kit. Primers used were as follows: actin (728 bp), forward 5’-AAG ATG ACC CAG ATC ATG TTT GAG AC-3’ and reverse 5’-TCA TGC CCT CCG GAC CCA CAT CCG CCT GAT CCA AAG ATG ACC CAG CAA CAC CAG GAC CCA AAG T-3’. Reactions were performed in an Eppendorf master cycler. One PCR cycle at 45°C for 45 min and 94°C for 2 min followed by forty PCR cycles under standard conditions with an annealing temperature of 60°C were performed. β-actin mRNA was used as a reference message to normalize the content of total RNA VEGF expression was calculated as the relative expression ratio to that of β-actin. All reactions were carried out in triplicate. Quantification was determined by triplicate repeats (26). Curcumin used for AP-1 inhibition studies was obtained from Sigma and prepared as a stock solution in Me2SO at 10 mg/ml.

Signal Transduction Array—The protein array was accomplished to compare the transcription factors involved in L10BIOBRMAPKK and L10BIOBRGFP cells. Nuclear extract was isolated from these cells using the Panomics nuclear extraction kit (catalog number AY2002), and the array was performed using TranSignal™ protein/DNA arrays. In brief, DNA/protein hybridization was carried out according to the manufacturer’s instructions. The gel area was excised containing the protein/DNA complex, and the protein/DNA complex was extracted using the extraction buffer and finally incubated with 6 μl of gel extraction beads at room temperature for 10 min. The mixture was centrifuged at 10,000 rpm for 30 s to pellet out the beads. The beads were washed, and the supernatant was removed. The bound probes were eluted by resuspending the pellet in 50 μl of dH2O and incubated at room temperature for 10 min with vortexing for two to three times during incubation.

Detection was done after the addition of 20 ml of 1× blocking buffer to each membrane and incubation at room temperature for 15 min with gentle shaking. 20 μl of streptavidin-horseradish peroxidase conjugate was added directly to 20 ml of 1× blocking buffer, and continued shaking at room temperature was conducted for 15 min. Each membrane was washed three times at room temperature with 20 ml of 1× wash buffer each 8 min followed by the exposure of 20 ml of 1× detection buffer to each membrane and incubation at room temperature for 5 min. 2 ml of a substrate working solution containing equal volumes of the luminol enhancer and peroxide solution was added, and the membranes were incubated at room temperature for 5 min and then exposed using Kodak BioMax film.

RESULTS

Active MAPK Is Found in Melanoma but Not in Benign Nevi—To determine whether phosphorylated (active) MAP kinase is activated in malignant melanoma, we performed immunohistochemical analysis of both benign melanocytic nevi and malignant melanoma according to the method of Arbiser et

![Fig. 3. MAPKK activation causes transformation in vivo. The left panel shows mice containing MAPKK-induced tumor (mouse on the left) and GFP control tumor (mouse on the right). The right panel shows that introduction of MAPKK into L10BIOBR cells leads to a significant difference in tumor volume at 1 month post inoculation (p < 0.05).](image)

![Fig. 4. A, effect of MAPKK activation on VEGF expression. The top panel demonstrates a representative RT-PCR analysis for VEGF in MAPKK-expressing and control L10BIOBR melanocytes. The left lane is a molecular weight standard, the middle lane represents RNA from MAPKK-expressing melanocytes, and the right lane represents RNA from control melanocytes. RNA samples were analyzed for β-actin expression as a loading control. B, effect of MAPKK activation on MMP bioactivity. The left lane represents triplicate samples of conditioned media from L10BIOBR-GFP (vector control) cells, and the right lane represents conditioned media from active MAPKK-expressing L10BIOBR cells. The lower band at 65 kDa indicates gelatinolysis induced by MMP-2, and the upper band at 92 kDa indicates gelatinolysis induced by MMP-9. C, effect of MAPKK on TIMP bioactivity. The left lane represents triplicate samples of conditioned media from L10BIOBR-GFP (vector control) cells, and the right lane represents conditioned media from active MAPKK-expressing L10BIOBR cells. The inhibitory activity of conditioned media from MAPKK-expressing cells was significantly higher than that of control GFP cells.](image)
We have previously demonstrated the specificity of this antibody using melanoma cell lysates (16, 18). Very little staining was observed in benign melanocytic nevi, but prominent nuclear staining was observed in human melanoma tumors (Fig. 1).

Introduction of Active MAPKK into Immortalized Melanocytes—The L10BIOBR cell line is an immortalized murine melanocyte cell line that has been used to assess tumorigenesis. To determine whether the activation of MAP kinase signaling results in tumorigenesis, we infected these cells with retroviruses encoding constitutively active MAPKK or GFP as a vector control. The introduction of active MAPKK led to an increase in total (endogenous and retrovirally transduced) MAPKK protein as assessed by Western blot (Fig. 2A). To determine whether the introduced MAPKK is functional, we examined levels of active (phosphorylated) MAPK in both cell lines. MAPK phosphorylation is significantly increased in L10BIOBR cells expressing active MAPKK (Fig. 2A). Melanocytes expressing active MAPKK showed increased refractility and elongated shape compared with control cells (Fig. 2B).

Melanocytes Expressing Activated MAPKK Are Tumorigenic in Vivo—To determine whether overexpression of MAPKK causes malignant transformation in melanocytes, one million cells of either vector control or MAPKK-expressing L10BIOBR cells were injected subcutaneously into nude mice. Overexpression of MAPKK leads to malignant transformation in vivo, whereas progressive tumor growth was not observed in vector expressing cells (Fig. 3).

Introduction of Active MAPKK Induces the Angiogenic Switch—Part of the action of dominant oncogenes is to activate the angiogenic switch toward cells that produce angiogenesis stimulators. We examined the production of VEGF mRNA by RT-PCR and found 5-fold induction by MAPKK (Fig. 4A). Substrate gel electrophoresis using gelatin as the substrate indicated that the introduction of MAPKK into melanocytes resulted in an increase in the gelatinase activity of MMP-9 (92 kDa) and MMP-2 (65 kDa) in comparison to control cells (Fig. 4A). Surprisingly, when this same conditioned media was tested in a radiometric enzyme assay for TIMP activity, we found that the MAPKK cells produced significantly higher levels of MMP inhibitory activity than did the control cells (Fig. 4C).

Analysis of Tumor Dormancy and Vascularity—In situ hybridization of vector control and MAPKK-expressing tumors for the proliferation-associated marker histone H3 revealed little expression of this marker in vector control dormant tumors but diffuse expression in MAPKK-expressing L10BIOBR tumors (Fig. 5, A–D). These results are similar to what we have observed with immortalized and transformed endothelial cells in that both three-dimensional growth and increased angiogenesis are required for tumor growth in vivo (14). MAPKK-overexpressing melanoma tumors also demonstrate a high level of vascularity consistent with elevated expression of VEGF. MAPKK-expressing tumors also were notable for a high level of vascularity compared with GFP vector controls (Fig. 5, E and F).

Effect of MAP Kinase Activation on AP-1 and NFκB—Both AP-1 and NFκB have been reported to be potential downstream mediators of MAP kinase signaling (27–29). To determine which pathway is downstream of MAPKK in melanocytes over-
expressing MAPKK, we performed a Panomics assay, which measures relative activation of transcription factors. In this assay, we observed activation of AP-1 but not NFκB (data not shown). We further demonstrated that activation of MAP kinase kinase in melanocytes led to phosphorylation of the AP-1 transcription factor c-Jun (Fig 6A). Finally, MAP kinase activation led to increased phosphorylation of p70 S6 kinase and its downstream target, S6 ribosomal protein (Fig 6B). Increased AP-1 activation was functionally important in melanocytes overexpressing MAP kinase kinase, because inhibition of c-Jun with the small molecular weight inhibitor curcumin (30–33) led to decreased production of VEGF mRNA in a dose-dependent manner (Fig 7). Curcumin has little effect on MMP activity in MAPKK-transformed L10BIOBR melanocytes, suggesting that a c-Jun-independent pathway mediates MAPKK activation of MMP activity (data not shown). We did not observe any effect of MAPKK on hypoxia-inducible factor-1α (HIF-1α) activation either (data not shown). Consistent with our findings that there is a lack of activation of NFκB in MAPKK-transformed L10BIOBR melanocytes, treatment of these cells with NFκB inhibitors (SN50, hypoestoxide) had no significant effect on VEGF mRNA levels (data not shown).

**Effect of MAP Kinase Activation on Phosphorylation of p70 S6 Kinase and S6—Activation of p70S6 kinase and S6 by phosphorylation has been implicated in the regulation of VEGF mRNA and protein synthesis (29, 34). To determine whether the activation of MAP kinase activated p70S6 kinase and S6, Western blot analysis using phospho-specific antibodies was performed. The activation of MAP kinase led to constitutive activation of p70S6 kinase and S6 (Fig. 8).**

**DISCUSSION**

Melanoma is a common malignant tumor with a rapidly increasing incidence. Although early melanoma is curable through surgical excision, the prognosis of advanced melanoma is dismal. Therefore, knowledge of the signal transduction pathways involved in melanoma genesis is crucial for the future treatment of melanoma. Most melanomas are thought to arise in precursors termed atypical nevi, and transition zones have been observed in atypical nevi, which show frank malignant changes (35). Melanomas progress to a stage called radial growth melanoma, which is characterized by the growth of tumors along the dermoepidermal junction, but without invasion. Further genetic changes convert melanoma into an invasive tumor capable of three-dimensional growth, increased angiogenesis, and metastasis (14, 36–38). Our findings in this model closely resemble the changes observed in human early radial growth melanoma (16) in which MAP kinase is activated and VEGF is induced. Other investigators have implicated NFκB in melanoma cells, but these studies have involved cell lines known to have aggressive behavior (39, 40). A comparison of vector control melanocytes with melanocytes overexpressing activated MAP kinase kinase reveals preferential activation of AP-1 rather than NFκB by MAPKK. In this system, MAPKK activation results in the activation of c-Jun and the induction of p70S6 kinase, resulting in the induction of VEGF. This pathway has also been described as a response to the angiogenic factor insulin-like growth factor 1 (IGF-1), which is also capable of causing MAP kinase activation and the subsequent induction of p70S6 kinase, hypoxia-inducible factor-1α, and VEGF (29,34). The data presented here support a stepwise progression, with activation of MAP kinase/AP-1 signaling being an initial step seen in early melanoma, followed by activation of NFκB associated with increased aggressive behavior (39–41).

Genetic changes commonly associated with melanoma include the loss of the tumor suppressor p16ink4a and the increased expression of the p16ink4a-suppressing gene Id-1 (42). Later changes in melanoma include activation of ras and the loss of the tumor suppressor PTEN (43, 44). Transgenic experiments have demonstrated a synergy between oncogenic ras and p16ink4a loss in the development of melanoma, and maintenance of melanoma is dependent upon the continuous presence of activated ras (45). However, the pathways downstream of ras necessary for melanoma growth in vivo are not known. The activation of ras or the loss of PTEN is capable of activating several signal transduction pathways, including MAP kinase signaling (46–50).
The introduction of MAPKK into melanocytes resulted in an increase in the levels of the gelatinase MMP-2. Interestingly, we also detected significantly higher levels of TIMP activity in this same conditioned media. Because the radiometric enzyme assay that was used to determine the MMP inhibitory activity measures levels of “free” TIMP activity, these results may suggest that the net proteolytic balance in these cells is shifted in favor of MMP inhibition. An inhibition of MMP by TIMP may account in part for the poorly invasive growth of the MAPKK-transformed tumor (51, 52).

Controversy exists over the role of MAP kinase in tumorigenesis (53). Initial studies have shown that MAP kinase activation can lead to the transformation of NIH3T3 cells but also lead to differentiation of PC12 cells (47, 55). We have previously demonstrated that inhibition of MAPK signaling with a dominant negative MAPKK leads to induction of MMP and does not inhibit tumorigenesis in a murine model of angiosarcoma (51). Consistent with our findings in mice, we have also demonstrated that the expression of activated MAPK decreases with increasing malignancy in human endothelial tumors (18). Thus, the role of MAP kinase activation in promoting tumorigenesis may be dependent upon the tissue and tumor suppressor context of a given tumor (4, 56). We felt that MAPK was a strong candidate in melanoma, because activated MAPK is expressed in melanoma but not benign nevi (54, 57). Our results suggest that inhibition of MAPK may be a thera- peutic target in melanoma. In addition, immunohistochemical analysis of a given tumor type with antibodies specific for active MAP kinase may help determine whether this gene is involved in the angiogenic switch in a given tumor type and help guide therapy.

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