Overexpression of Collagenase 1 (MMP-1) Is Mediated by the ERK Pathway in Invasive Melanoma Cells

ROLE OF BRAF MUTATION AND FIBROBLAST GROWTH FACTOR SIGNALING*

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Melanoma progresses as a multistep process where the thickness of the lesion and depth of tumor invasion are the best prognostic indicators of clinical outcome. Degradation of the interstitial collagens in the extracellular matrix is an integral component of tumor invasion and metastasis, and much of this degradation is mediated by collagenase-1 (MMP-1), a member of the matrix metalloproteinase (MMP) family. MMP-1 levels increase during melanoma progression where they are associated with shorter disease-free survival. The Ras/Raf/MEK/ERK mitogen-activated protein kinase (MAPK) pathway is a major regulator of melanoma cell proliferation. Recently, BRAF has been identified as a common site of activating mutations, and, although many reports focus on its growth-promoting effects, this pathway has also been implicated in progression toward metastatic disease. In this study, we describe four melanoma cell lines that produce high levels of MMP-1 constitutively. In each cell line the Ras/Raf/MEK/ERK pathway is constitutively active and is the dominant pathway driving the production of MMP-1. Activation of this pathway arises due to either an activating mutation in BRAF (three cell lines) or autocrine fibroblast growth factor signaling (one cell line). Furthermore, blocking MEK/ERK activity inhibits melanoma cell proliferation and abrogates collagen degradation, thus decreasing their metastatic potential. Importantly, this inhibition of invasive behavior can occur in the absence of any detectable changes in cell proliferation and survival. Thus, constitutive activation of this MAPK pathway not only promotes the increased proliferation of melanoma cells but is also important for the acquisition of an invasive phenotype.

Melanoma, like other malignant cancers, progresses as a multistep process that can be classified histologically, with the depth of tumor invasion being the best prognostic indicator of clinical outcome (1–3). Invasive melanomas are clinically aggressive and prone to metastasis, leading to disseminated disease (4, 5). Multiple stages leading to metastatic disease have been described; however, the transition from the radial growth phase (RGP)1 to the vertical growth phase (VGP) stands out as a particularly biologically and clinically important step (3). As a rule, RGP melanomas grow in a plane parallel with the skin’s surface and are not capable of metastasizing. Largely, they remain within the epidermis, and have only very limited dermal infiltration. In contrast, VGP melanomas grow perpendicular to the skin’s surface and are able to metastasize. They can invade deeply into the underlying dermis, which is composed of numerous structural obstacles, the most prevalent being type I collagen. The molecular events underlying the acquisition of vertical growth and of a metastatic phenotype are of obvious importance and are being actively investigated (3, 6–8).

Tumor invasion and metastasis require proteolytic degradation of the basement membrane and extracellular matrix (ECM) by the matrix metalloproteinases (MMPs). MMP expression is low in most normal cells under physiologic conditions, however, MMP expression is dramatically increased in a variety of cancer types, where it is indicative of invasive disease with a poor clinical prognosis (4, 5, 9–12). One MMP family member capable of degrading the most abundant proteins of the ECM (collagen types I and III) is the interstitial collagenase, MMP-1. MMP-1 is overexpressed in invasive melanoma (5, 9) and is required for melanoma cell invasion through a synthetic ECM in vitro (13).

In normal cells, MMP-1 expression can be induced by a variety of growth factors, including bFGF, epidermal growth factor, interleukin-1, and tumor necrosis factor α (14). This induction requires the activation of the mitogen-activated protein kinase (MAPK) pathways, which in turn act in part through activation of AP-1 and ETS family transcription factors (14–19). There are at least three major human MAPK families: the extracellular response kinases (ERKs), p38, and...

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The abbreviations used are: RGP, radial growth phase; bFGF, basic fibroblast growth factor; DMEM, Dulbecco’s modified Eagle’s medium; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; FGFR, fibroblast growth factor receptor; FGFR-DN, FGFR dominant negative; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; JNK, Jun N-terminal kinase; LH, lactalbumin hydrolysate; MMP, matrix metalloproteinase; MAPK, mitogen-activated protein kinase; VGP, vertical growth phase; MEK, MAPK/ERK kinase; HBSS, Hank’s balanced salt solution; RT, reverse transcriptase; RLU, relative light unit(s).
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The dominant negative RAF (Raf-N4) encodes the tandem Raf-binding domains (RBD and CBD; residues 23–284) of c-Raf in the expression vector pGCM-hygro (45), and the constitutively active human BRAF (V599E) construct was cloned into the pBABE Puro expression plasmid.²

Cell Culture—A2058 cells were purchased from ATCC. VM55, VMMD12, and VMMD39 melanoma cells were initially isolated from lymph node metastasis from three separate patients at the University of Virginia, Charlottesville, VA. The cells were propagated in 150-mm diameter culture dishes in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) containing 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) (37 °C in 5% CO₂). After 3–4 days, when the cells were confluent, they were passaged 1:10 with 0.25% trypsin. In experiments performed under serum-free conditions, cells were grown to the desired level of confluence, washed with Hanks’ balanced salt solution (Invitrogen) to remove traces of serum, and placed in serum-free DMEM supplemented with 0.2% lactalbumin hydrolysate.

Western Blot Analysis—For analysis of total and phosphorylated MAPK proteins, duplicate wells of melanoma cells were washed with Hanks’ balanced salt solution and then scraped in 2× denaturing SDS buffer. Extracts were heated for 10 min at 100 °C and resolved by SDS-PAGE and immunoblotted as described previously (16, 18). Antibodies to p38, pp38, MEK1/2, ppMEK1/2, pp42/44, and p42/44 were purchased from Cell Signaling.

Northern Blot Analysis—Confluent cultures of melanoma cells were placed in serum-free medium for 24 h. Total RNA was harvested with TRIzol (Invitrogen). RNA (10 μg/lane) was subjected to electrophoresis on 1% formaldehyde agarose gels and transferred to a GeneScreen Plus membrane. Membranes were hybridized with denatured (α-32P) dCTP dNAs for MMP-1, labeled by random oligonucleotide primer, and exposed to x-ray film overnight.

Real-time RT-PCR—Total cellular RNA was harvested using the RNeasy kit (Qiagen) and subjected to on-column DNase treatment as per the manufacturer’s instructions. RT and real-time PCR were performed using protocols and reagents from Applied Biosystems Taqman RT reagent kit and Sybr Green PCR master mix as described (16). Briefly, 2 μg of DNase-treated RNA was reverse-transcribed in a 20-μl reaction containing 5.5 mM MgCl₂, 500 μM each dNTP, 2.5 μM oligonucleotide d(T)₁₆, 0.4 unit/μl RNase inhibitor, and 1.25 unit/μl Multiscribe reverse transcriptase. The reactions were incubated at 25 °C for 5 min, 48 °C for 30 min, and then 95 °C for 5 min. Five microliters of each RT reaction was used to amplify each mRNA in triplicate real-time PCR reactions. To enable quantitative comparisons between PCR assays, standard curves were generated with every assay. Serial log dilutions ranging from 1 ng to 100 fg of American Type Culture Collection plasmids pSP6-MMP-1 and pCMV sport6 were used as standards for MMP-1 and GAPDH, respectively. Sequences for the MMP-1 primers were 5'-GACGTCGACGAGCATGATG-3' (sense) and 5'-GGCGTGATTCAACAGGCACGAGC-3' (antisense); and GAPDH primers were 5'-GGACGAGAGGCTGACCTT-3' (sense) and 5'-CCGGATGCAGTTC-3' (antisense). The PCRs contained 200 nt of each primer and were incubated on a Molecular Dynamics Opticon thermal cycler at 95 °C for 10 min, followed by 40 PCR cycles of 95 °C for 15 s, and 60 °C for 1 min, and a plate read. The PCR cycles were followed by a Sybr green melting curve from 55 to 90 °C. MMP-1 mRNA expression was normalized to GAPDH mRNA expression and is reported as mean copies of MMP-1 message per picogram of GAPDH.

Transient Transfections—Triplicate cultures of melanoma cells were plated at a density of 3 × 10⁵ cells per well in 6-well tissue culture plates and transfected with effectamine 2000 vector reagent (Invitrogen) as per the manufacturer’s instructions. The next day, cells were placed in serum-free medium, and in some experiments, treated with MAPK inhibitors or MeSO vehicle control. After 8 h, cells were washed three times with phosphate-buffered saline and lysed in 1× Passreve Lysis Buffer (Promega). Luciferase activity was measured and reported as relative light units. For co-transfections with Depot MAPK mutants, the total amount of DNA was kept constant by the addition of the appropriate empty vector control, and the mean of the empty vector control values for each cell line was set arbitrarily at 100 to facilitate the graphical display of multiple cell lines in the same chart.

Fibrillar collagen was prepared from bovine type I collagen (Vitrogen 100, Cohesion Technologies, Palo Alto,

²H. Hao, V. M. Muniz-Medina, K. Frantz, H. Mehta, N. E. Thomas, C. J. Der, and J. M. Shields, manuscript in preparation.
CA) as described by the manufacturer. Briefly, the collagen was neutralized by the addition of 10× phosphate-buffered saline and 0.1 N NaOH until the final pH reached 7.4. Next, 2×10^6 melanoma cells suspended in 0.5 ml of serum-free DMEM were mixed with 0.5 ml of the neutralized collagen solution and added to one well of a 6-well plate (final collagen concentration is 1.2 mg/ml). Plates were then transferred to a 37 °C tissue culture incubator for at least 1 h to promote collagen gel formation. One ml of serum-free DMEM plus any treatment was then added to the collagen gel (~1 mm thick) with embedded cells. After 48 h, the overlying medium was removed and weighed. Finally, side-view photographs of the remaining collagen gel were taken. All experiments were done in triplicate.

Cell Number Assays—Parallel cultures of melanoma cells (350,000 cells per well) were plated in DMEM with 10% serum and allowed to adhere overnight. After 16 h, the cells were washed and switched to serum-free DMEM with U0126 (5 μM) or MeSO. Cells were harvested by trypsin digestion and counted at the times indicated. All experiments were performed in triplicate.

Statistical Analysis—All numerical values reported represent mean ± S.D. Statistical significance compared with control values was calculated using the Student’s t test available at www.physics.csbsju.edu/stats/. Asterisks were used to graphically denote statistical significance (p < 0.05) in the figures.

RESULTS

Inhibition of MAPK Pathways Decreases the High Constitutive Expression of MMP-1 in VMM and A2058 Melanoma Cells—As a first step in defining the molecular mechanisms underlying MMP-1 expression in invasive melanoma, we chose four melanoma cell lines and measured MMP-1 mRNA production in each cell line by Northern blot analysis (Fig. 1A) and real-time RT-PCR (Fig. 1B). In each cell line, MMP-1 mRNA expression was constitutively high, and greatly exceeded the level of expression of MT1-MMP, MMP-2, MMP-3, MMP-9, and MMP-13 (Ref. 16 and data not shown). We have previously reported that ERK is constitutively active in the VMM5 and A2058 cells, and that MMP-1 expression is decreased in cells treated with an inhibitor of the ERK pathway (16, 19, 46). To investigate if this observation could be extended to the other melanoma cells, we first measured ERK activity in VMM12 and VMM39 cells cultured in serum-free conditions and found that both cell lines display constitutively active ERK, which can be inhibited by treatment with 5 μM of the MEK1/2 specific inhibitor U0126 (Fig. 1C).

Next we treated cells with U0126 (5 μM) and measured MMP-1 mRNA by real-time RT-PCR 18 h later. Fig. 2A shows a statistically significant (p < 0.0001) decrease (70%) in MMP-1 mRNA levels in both the VMM12 and VMM39 cells after U0126 treatment. Since we have previously reported that VMM5 melanoma cells also rely on the p38 pathway for MMP-1 production (16), we investigated whether the other VMM melanoma cell lines utilize this pathway as well. Fig. 2B shows dose-response curves of MMP-1 mRNA levels in VMM12 and VMM39 cells treated with the p38 inhibitor SB203580. Similar to the VMM5 cells (16), VMM12 cells show a significant dose-dependent decrease in MMP-1 mRNA when treated with 5 and 10 μM SB203580, whereas the VMM39 cells show a modest but significant (p < 0.03) induction of MMP-1. These changes in mRNA were mirrored in secreted MMP-1 protein levels as measured by Western blot analysis of precipitated culture medium (Fig. 2C). To determine if the effects of U0126 and SB203580 occurred at the level of gene transcription, we transiently transfected both the VMM12 and VMM39 cells with a luciferase reporter construct driven by 4.4 kb of the human MMP-1 promoter. Both inhibitors decreased MMP-1 promoter activity in the VMM12 cells, while U0126 decreased, and SB203580 increased MMP-1 in the VMM39 cells (Fig. 2D). Similar to previous studies with the VMM5 and A2058 cells (16, 19, 46), these findings parallel those obtained for the endogenous gene, arguing that both U0126 and SB203580 exert their inhibitory effects at the level of transcription.

SB203580 Decreases MEK and ERK Activity in VMM5 and VMM12, but Not VMM39 Cells—Because considerable crosstalk occurs among different MAPK pathways (25, 47–49), we investigated the effect of p38 inhibition on the activation of MEK and ERK. Each VMM cell line was treated with 10 μM SB203580 for 18 h, and protein lysates were subjected to Western blot analysis for phosphorylated (active) MEK1/2 and ERK1/2 as well as total ERK1/2. In each cell line, MEK and ERK are constitutively active (Fig. 3). In the VMM39 cells, SB203580 treatment induced ERK1/2 and MEK1/2 activity, consistent with other published reports that the p38 pathway can act as a negative regulator of MEK/ERK signaling (48, 49). However, in the VMM5 and VMM12 cells, the opposite effect was observed: SB203580 caused a substantial decrease in phospho-MEK and ERK levels (Fig. 3A). In the VMM12 cells, this inhibitory effect was rapid (within 15 min of treatment), prolonged (significant up to 25 h) (Fig. 3B), and was observed at concentrations as low as 5 μM (data not shown).

Sodium Arsenite Activation of ERK, but Not p38, Induces MMP-1 mRNA in VMM Cells—Given the inhibitory effects of SB203580 on the ERK pathway in VMM5 and VMM12 cells, it is possible that SB203580 decreased MMP-1 production indirectly through its effects on MEK/ERK. To test this hypothesis, we investigated whether MMP-1 production could be induced by ERK and/or p38 activation. One agent known to induce
multiple MAPK pathways, including p38 and ERK, is sodium arsenite (25, 49–51). Therefore, we treated the VMM12 and VMM39 cells with 20 μM sodium arsenite and measured the time course of both ERK and p38 activation. Phospho-p38 was virtually undetectable in the untreated cells but increased substantially in the first hour after arsenite treatment (Fig. 4A). Arsenite treatment also strongly induced ERK phosphorylation in both cell lines within 30 min (Fig. 4A). Induction of both p38 and ERK was sustained, persisting for at least 8 h. Based on these results, we next treated each cell line with 20 μM sodium arsenite and measured MMP-1 mRNA levels by real-time RT-PCR after 8 h. To assess the contribution of each MAPK pathway, we also included experimental groups treated with the p38 inhibitor SB203580 (10 μM) and the MEK inhibitor U0126 (5 μM). We found that arsenite induces MMP-1 mRNA in both the VMM12 (Fig. 4B) and VMM39 cells (Fig. 4C), and this induction can be blocked by the addition of U0126. However, treatment with SB203580 cannot block the arsenite-mediated induction of MMP-1 in either cell line. Taken together, these data show that the MEK/ERK pathway, but not the p38 pathway, is capable of inducing MMP-1, and argue in favor of ERK playing a dominant role in the constitutive production of MMP-1 in these cells.

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Overexpression of Constitutively Active BRAF but Not MKK3 Activates MMP-1 Promoter Activity in VMM Melanoma Cells—To further analyze the ability of the p38 and ERK pathways to induce MMP-1, we overexpressed two constitutively active upstream components of these two pathways (MKK3 and BRAF) by transient transfection and measured MMP-1 promoter activity. When constitutively active MKK3, an upstream inducer of p38 activity, was overexpressed, it had no effect on MMP-1 in the VMM12 cells at any concentration tested (Fig. 5A). In the VMM39 cells, only the highest concentration (1 μg) had a modest inhibitory effect (Fig. 5A), illustrating that, in these cells, p38 activity is actually a negative regulator of MMP-1. In contrast, activating the MEK/ERK pathway by overexpressing an activated BRAF mutant significantly increased MMP-1 promoter activity in a dose-depend-
arsenite (20 μM) for 8 h. At this time, the cells were lysed in passive lysis buffer, and luciferase activity was measured. All experiments were done in triplicate and reported as mean RLU ± S.D. *p < 0.05 compared with Me 2SO plus vehicle control for 8 h indicated. Total RNA was isolated, and MMP-1 mRNA levels were measured by real-time RT-PCR. Values are reported in percentage of Me 2SO control (mean ± S.D.). #p < 0.05 compared with the V599E BRAF mutant, c-RAF4N overexpression had no significant effect on MMP-1.

Constitutive ERK activation may also arise due to autocrine growth factor production by melanoma cells (30). One factor that has received considerable attention is basic fibroblast growth factor (bFGF) (53–57), a potent inducer of the Ras/Raf/MEK/ERK pathway and MMP-1 transcription in other cell types (15, 21). Thus, we tested whether MMP-1 production in the VMM39 cells was dependent on FGF signaling by overexpressing a truncated, dominant negative form of the FGF receptor (FGFR-DN) and measuring MMP-1 promoter activity. Fig. 6B shows that with increasing amounts of FGFR-DN, there is a dose-dependent decrease in MMP-1 promoter activity in the VMM39 cells, arguing that in these cells MMP-1 production is dependent on autocrine FGF signaling. In contrast, no inhibition of MMP-1 is seen in the V599E BRAF-positive VMM5 or VMM12 cells. Interestingly MMP-1 is actually induced with FGFR-DN overexpression in the VMM12 cells indicating that in these cells autocrine FGF signaling exerts a negative influence on MMP-1 expression. The signaling events contributing
to this negative regulation are not known, and given that VMM12 cells continue to express MMP-1 at high levels despite the presence of this autocrine, inhibitory effect, we believe that it is of relatively little functional importance.

**MEK Inhibition Decreases Both Melanoma Proliferation and Collagenolytic Activity in Vitro**—To investigate whether the U0126-mediated decrease in MMP-1 expression was functionally significant, we chose the two cell lines exhibiting the highest MMP-1 production (A2058 and VMM12) and measured their ability to digest a synthetic type I collagen matrix in vitro. After embedding in fibrillar collagen, A2058 and VMM12 cells were treated with U0126 or Me2SO vehicle control and incubated at 37 °C for 48 h. Fig. 7A shows that neither A2058 nor VMM12 cells treated with U0126 were able to digest the type I collagen, whereas little or no collagen remained in the wells treated with Me2SO. We quantified the amount of collagen digested by collecting and weighing the culture medium from each well. Because 1 ml (1 g) of medium was added to each well at the time of collagen gel composition, the recovery of more than 1 g of medium indicates that collagen breakdown has occurred due to the liberation of medium, which had previously been contained within the collagen gel. Fig. 7B shows the mean weight of medium collected for each cell line treated with U0126 or Me2SO. In both cell lines, 1 ml (1 g) of medium was recovered from wells treated with U0126, whereas greater quantities were recovered from wells treated with Me2SO, indicating that MEK/ERK inhibition resulted in significant (p < 0.0001 for A2058; p = 0.002 for VMM12) abrogation of collagen degradation.

Because the RAF/MEK/ERK pathway has been implicated in melanoma survival and proliferation (29, 36, 40), we examined the effect of MEK inhibition on cell growth. Equal numbers of VMM5, VMM12, VMM39, and A2058 cells were plated in serum-containing medium. After 16 h, the cells were switched to serum-free DMEM and treated with either Me2SO or U0126 and allowed to grow for an additional 48 h. Cells from each well were trypsinized and counted. Fig. 7C shows the cell numbers for each cell line treated with Me2SO vehicle or U0126. Both the VMM5 and VMM12 cell numbers were significantly (p < 0.001) affected by MEK inhibition, with each cell line showing ~75% fewer cells after 48 h of treatment. In contrast, VMM39 and A2058 cell proliferation were largely unaffected, suggesting that other growth-promoting pathways are also active in these cells. It is noteworthy that, in the A2058 cells, MEK/ERK inhibition by U0126 completely prevented collagen digestion yet had no significant effect on cell survival or proliferation.

Because U0126 treatment decreased the cell number in the VMM12 cells, and this difference could influence collagenolytic activity as measured by our degradation assay, we examined the effect of U0126 treatment on cell proliferation more closely. Fig. 7D shows the kinetics of the change in VMM12 cell number after treatment with either Me2SO or U0126. At the start of treatment, each well averaged ~300,000 cells, and this number was not significantly changed in either treatment group after 24 h. After 48 h of treatment, there were ~440,000 more cells in the Me2SO-treated wells as compared with the U0126 wells (605,000 and 164,000, respectively), however, most of this difference arose due to the continued proliferation of Me2SO-treated cells (net increase of 325,000 cells) as compared with the death of U0126-treated cells (net decrease of 116,000 cells). Although we can clearly not comment on the mechanism underlying the decrease in cell number after U0126 treatment, it stands to reason that if the inhibitory effect of U0126 on collagen degradation was due to the decreased number of cells, rather than to a decrease in MMP-1 production, simply plating more cells in the U0126-treated wells should result in significant collagen degradation. However, this was found not to be the case, because collagenolytic activity was not restored in U0126-treated cells even when the number of cells was increased 4-fold (Fig. 7E). Thus, the decrease in collagen degradation by U0126 treatment can not be explained simply by its effects on cell number and most likely is a direct result of U0126 inhibition of MMP-1 production.

**DISCUSSION**

In melanoma, multiple stages leading to metastatic disease have been described, however, the transition from nonmetastatic RGP to metastatically competent VGP stands out as a particularly important step, biologically and clinically (3). Because MMPs are critical facilitators of tumor invasion, their expression patterns have been studied during different stages of melanoma progression and found to be temporally and spatially regulated (9, 12). These expression patterns suggest that specific MMPs contribute to specific stages in melanoma progression (reviewed in Ref. 10) and that inhibiting MMP-1 production in melanoma cells decreases the invasive potential of melanoma cells (13), we have begun to investigate the molecular pathways underlying the constitutive expression of MMP-1 by melanoma cells (16, 19).

In the present study we report that MMP-1 production in four different melanoma cell lines depends upon the activity of the RAF/MEK/ERK MAPK pathway. Treating these cells with the MEK inhibitor U0126 decreases both MMP-1 mRNA and protein levels. Because U0126 also decreases MMP-1 promoter activity, this inhibition is most likely occurring at the level of transcription. Conversely, overexpression of an activated mutant of B Raf (V599E) further induces MMP-1. Inhibiting MEK/ERK activity and MMP-1 production with U0126 completely prevented collagen digestion by both the VMM12 and A2058 cells embedded in type I collagen. These results parallel those reported in another study in which melanoma cell invasion through Matrigel was found to be decreased with MEK/ERK inhibition (58) and underscore the importance of this MAPK pathway in the development of an invasive phenotype.
Our initial data indicated that MMP-1 production is decreased due to p38 inhibition by SB203580 (16). We can now report that the p38 pathway, when activated by either sodium arsenite or overexpression of an activated mutant of MKK3, is unable to induce MMP-1. Thus, the effect of SB203580 is most likely indirect, given that SB203580 treatment also decreases MEK/ERK activity. Whether this inhibition is a direct effect of SB203580 on MEK/ERK, or an indirect effect arising due to p38 inhibition remains to be determined; however, there are reports of SB203580 acting as a modulator of RAF activity (59, 60).

Our finding that constitutive MMP-1 production and MEK/ERK activity are associated with either an activating mutation in exon 15 of the \textit{BRAF} gene or autocrine FGF signaling is consistent with previously published reports (29, 30). It is interesting to note that, in our small sampling of melanoma cell lines, the three lines that have the \textit{V599E} \textit{BRAF} mutation (A2058, VMM5, and VMM12), all produce substantially more MMP-1 than the VMM39 cells, which rely on autocrine FGF signaling. Furthermore, when the \textit{V599E} \textit{BRAF} mutant is over-expressed in the VMM39 cells, MMP-1 induction is more robust, with a greater relative -fold induction than in either the VMM5 or VMM12 cells. Taken together, these observations suggest that direct activation of MEK is a more efficient means of activating MEK/ERK and its downstream effectors than indirectly activating the pathway via an autocrine growth factor loop, and may help to explain why such a high percentage of melanomas harbor \textit{BRAF} mutations.

In this study we focused on MMP-1, because it was the most abundantly expressed MMP with activity against the type I and III collagen molecules found in the dermal ECM. Each of the cell lines also produced MT1-MMP, a MMP that has been implicated in playing an important role in collagenolysis and tumor cell invasion (61). Although MT1-MMP expression has also been shown to depend on MEK/ERK activity in other

**Fig. 7.** MEK/ERK inhibition by U0126 decreases VMM cell proliferation and collagenolytic activity. A2058 or VMM12 melanoma cells were embedded in purified bovine type I collagen and 1 ml of serum-free DMEM with either Me2SO or U0126 (5 \mu M) was added to each well. After 48 h, all overlying medium was removed and weighed, and the underlying collagen gel was photographed. A, side-view image of a tissue culture plate showing collagen gel thickness after 48 h for A2058 (upper panel) and VMM12 cells (lower panel). Arrows indicate the lower and upper gel boundaries. B, mean weight (in grams ± S.D.) of overlying medium. Greater than 1 g (1 ml) of recovery indicates collagen degradation has occurred due to the liberation of medium from the gel. C, equal numbers of each cell line were plated in serum-containing DMEM and allowed to adhere overnight. The next morning, triplicate wells were switched to serum-free DMEM treated with U0126 (5 \mu M) or Me2SO. After 48 h, the cells were harvested by trypsin digestion and counted (values are mean number of cells ± S.D.). D, VMM12 cells were plated in triplicate in DMEM with 10% serum and allowed to adhere overnight. After 16 h, the cells were washed and switched to serum-free DMEM with U0126 (5 \mu M) or Me2SO. Cells were harvested by trypsin digestion and counted at the times indicated. Values reported are the mean number of cells per well ± S.D. E, VMM12 cells were embedded in collagen at three different densities (1X, 2X, and 4X) and treated with either U0126 (5 \mu M) or Me2SO as indicated. After 48 h, the overlying medium was removed and weighed. Values are mean weight in grams ± S.D.
tumor cell types (62), we observed no inhibition of MT1-MMP expression upon U1026 treatment (data not shown). Although we can not comment on the overall contribution of MT1-MMP to facilitating melanoma cell invasion, in this study, changes in type I collagenolytic activity did not correlate with changes in MT1-MMP expression.

Given our previous observations regarding the crucial role of stromal-derived proteases in facilitating melanoma invasion when A2058 cells were plated on top of collagen gels (63), we were intrigued by the ability of both the A2058 and VMM12 when A2058 cells were plated on top of collagen gels (63), we observed that the A2058 and VMM12 cells to digest type I collagen in the absence of either stromal cells or conditioned medium when directly embedded in a collagen matrix. This robust digestion of the collagen gel by the embedded cells (Fig. 7), but not the surface-plated cells (63), underscores the differences between a two-dimensional (cells plated on top) and a three-dimensional (cells embedded) environment. Identifying the factors induced by this three-dimensional environment, and the signaling mechanisms underlying this induction are currently being addressed.

The question of when BRAF mutations occur during melanoma progression continues to be an area of active investigation (32–35, 37–39, 52, 64–66), yet some common themes are emerging. BRAF mutations are more frequently found in metastatic disease as compared with primary melanoma and despite reports implicating BRAF mutation as an important indicator of melanoma transformation and proliferation (31, 36), several studies have concluded that BRAF mutation correlates most closely with melanoma progression and the development of metastasis (37–39, 64). Our findings are consistent with this conclusion, given our data showing that U1026 treatment significantly affects the cell number in only two of the four melanoma cell lines we examined, but decreases MMP-1 production in all four cell lines (Fig. 1 and Ref. 16). Even in cases when MEK/ERK inhibition does not decrease cell proliferation (such as in the A2058 cells), U1026 treatment abrogates the ability of these cells to digest collagen, and thus, may decrease their malignant potential.

In summary, we demonstrated that, in four different melanoma cell lines, the expression of MMP-1, a protein implicated in melanoma invasion and progression, relies upon MEK/ERK activity either due to BRAF-activating mutations or autoimmune FGF signaling. Inhibition of this activity significantly reduces MMP-1 expression and blocks collagenolytic activity. These data begin to provide one possible molecular link between the high frequency of BRAF mutation, constitutive ERK activity, and the development of a metastatic phenotype during melanoma progression.

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