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Phosphoinositide 3-Kinase Regulates Membrane Type 1-Matrix Metalloproteinase (MMP) and MMP-2 Activity during Melanoma Cell Vasculogenic Mimicry[1]

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Abstract

Vasculogenic mimicry (VM) describes the unique ability of highly aggressive melanoma tumor cells to express endothelial cell-associated genes (such as EphA2 and VE-cadherin) and form vasculogenic-like networks when cultured on a three-dimensional matrix. VM has been described in several types of aggressive tumors, including melanoma, prostate, breast, and ovarian carcinomas. However, the molecular underpinnings of this phenomenon remain somewhat elusive. In this study, we examined key molecular mechanisms underlying VM in aggressive human cutaneous and uveal melanoma. The data reveal that phosphoinositide 3-kinase (PI3K) is an important regulator of VM, specifically affecting membrane type 1 matrix metalloproteinase (MT1-MMP) and matrix metalloproteinase-2 (MMP-2) activity, critical in the formation of vasculogenic-like networks. Using specific inhibitors of PI3K, melanoma VM was abrogated coincident with decreased MMP-2 and MT1-MMP activity. Furthermore, inhibition of PI3K blocked the cleavage of laminin 5γ2 chain, resulting in decreased levels of the γ2′ and γ2x promigratory fragments. Collectively, these results indicate that PI3K is an important regulator of melanoma VM directly affecting the cooperative interactions of MMP-2, MT1-MMP, and laminin 5γ2 chain and, thus, the remodeling of the tumor cell microenvironment. PI3K may represent an excellent target for therapeutic intervention of a novel signaling cascade underlying VM.

Introduction

VM[3] describes the unique ability of highly aggressive human melanoma cells, but not poorly aggressive melanoma cells, to form matrix-rich networks *de novo* when cultured on a three-dimensional matrix, thus mimicking embryonic vasculogenesis (1). Recently, VM has been described in several other aggressive tumor types, including breast, prostate, and ovarian carcinoma, and may serve as an important target for new cancer therapies (2–10). Angiogenesis, or the signaling of new vessel growth from preexisting vessels, has been widely accepted as a means for tumor perfusion, resulting in growth and metastasis (11–13). Indeed, there has been an intense focus on targeting angiogenic, endothelial-lined blood vessels in an attempt to decrease tumor burden and ameliorate the disease. However, recent findings showing a physiological relationship between tumor cell VM and angiogenesis-related microcirculation (2, 6, 7, 11, 14, 15) may represent a more therapeutic opportunity for treating various aggressive tumor types.

The precise molecular events underlying the process of VM displayed by highly aggressive melanoma cells remain poorly understood. Our laboratory has begun to dissect this process using various strategies that have led to the identification of several key signal transduction molecules critical for VM (16, 17). Recently, MMP-2 and MT1-MMP/MMP-14 were identified as important mediators of VM (18). This study documented that these MMPs are important for cleavage of the laminin 5γ2 chain into promigratory fragments, essential in the formation of vasculogenic-like networks by melanoma cells. Others have reported that PI3K is important for angiopoietin-1-mediated endothelial cell sprouting by regulating MMP-2 (19) critical for angiogenesis. On the basis of these observations, we sought to investigate the potential role of PI3K as a mediator of VM.

In this study, we tested the hypothesis that PI3K regulates VM by mediating the activities of MT1-MMP and MMP-2 and ultimately the cleavage of laminin 5γ2 chain. Addition of LY294002, a specific inhibitor of PI3K, inhibited the ability of aggressive uveal and cutaneous melanoma cells to engage in VM on three-dimensional type I collagen matrices and to invade a defined matrix *in vitro*. Furthermore, addition of these inhibitors decreased the levels of active MMP-2 (as measured by zymography) and the expression and activity of MT1-MMP (as measured by real-time RT-PCR and ELISA assays) in a reversible manner. Moreover, Western blot analyses revealed a decrease in the levels of the promigratory laminin 5γ2′ and laminin 5γ2x fragments after inhibition of PI3K. Taken together, these results implicate PI3K as a key regulator of melanoma VM by mediating the activation of MT1-MMP and MMP-2 concomitant with the cleavage of laminin 5γ2 chain, which may serve as new molecular targets for therapeutic intervention of the signaling cascade underlying this unique process.

Materials and Methods

Cell Culture. The human cutaneous (C8161) and human uveal (MUM-2B and C918) melanoma cell lines have been previously described (20–22) and were maintained in RPMI 1640 (Invitrogen Life Technologies, Inc., Carlsbad, CA) supplemented with 10% fetal bovine serum and 0.1% gentamicin sulfate (Gemini Bioproducts, Calabasas, CA). Cell cultures were determined to be free of *Mycoplasma* contamination using the GenProbe rapid detection system (Fisher, Itasca, IL).

Three-Dimensional Cultures. Type I collagen three-dimensional matrices were prepared as described previously (17). Cells were allowed to adhere to the matrix for 1 h, at which time the media was removed, and fresh media containing LY294002 (20 μM final concentration in DMSO; Sigma Chemical Co., St. Louis, MO) was added. Control and recovery cultures received an equivalent volume of DMSO. Fresh media containing LY294002 was added every 24 h. After treatment, the cells were fixed and stained with Periodic Acid Schiff (17). Images were captured using an Axioskop 2 (Carl Zeiss, Inc., Thornwood, NY) and Spot 2 Camera (Diagnostic Instrument, Inc., Sterling Heights, MI) using the Zeiss Axiovision 2.0.5 software (Carl Zeiss, Inc.).

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[3] The abbreviations used are: VM, vasculogenic mimicry; MMP, matrix metalloproteinase; MT1-MMP, membrane type 1 MMP; PI3K, phosphoinositide 3-kinase; RT-PCR, reverse transcription-PCR; TIMP-2, tissue inhibitor of matrix metalloproteinase 2; PAS, periodic acid Schiff.

Electrophoresis and Immunoblotting. Cell lysates from MUM-2B, C8161, and C918 cultured on three-dimensional type I collagen (untreated or treated with LY294002) were collected in a manner described previously (17). Protein concentrations were determined using a BCA Protein Assay Reagent kit (Pierce Corp., Rockford, IL). Fifteen-50 μg of whole cell lysates were separated by 10 or 7.5% (for laminin 5γ2 chain) SDS-PAGE and transferred to nitrocellulose (Schleicher and Schuell, Keene, NH). Blots were blocked with 5% nonfat milk in TBS-TB (0.05% Tween 20; 0.05% BSA) for 1 h at room temperature. Blots were then incubated with anti-AKT(pSer^{473}; 1:1000; Biosource, Camarillo CA), anti-AKT (1:1000; Upstate, Lake Placid, NY), or anti-laminin 5γ2 chain (5 μg/ml; Chemicon International, Temecula, CA) followed by incubation with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary (1:5000; The Jackson Laboratory, Bar Harbor, ME). Blots were developed using an enhanced chemiluminescence detection kit (Perkin-Elmer Life Sciences Inc., Boston, MA).

Real-Time RT-PCR. RNA was extracted from MUM-2B, C8161, and C918 cells cultured on a type I collagen three-dimensional matrix and either left untreated (DMSO control) or treated with 20 μM LY294002, using Trizol reagent (Invitrogen Life Technologies, Inc.) following the manufacturer's protocol. Real-time PCR primers (forward 5′-GCCTGCGTCCATCAA-CACT-3′ and reverse 5′-ACACCCAATGCTTGTCTCCTTT-3′; Integrated DNA Technologies, Coralville, IA) and probe (5′-CCTACGAGAGGAAG-GATGGCAAATTCGTCT-3′; Integrated DNA Technologies) were designed using Primer Express Software (PE Applied Biosystems, Boston, MA) from 3558 bp of human MT1-MMP sequence. The probe was modified with 5′ reporter dye 6-carboxyfluorescein and the 3′ quencher dye 6-carboxy-*N*,*N*,*N*′,*N*′, tetramethylrhodamine. Real-time RT-PCR analyzed each RNA extract in triplicate with the TaqMan one-step RT-PCR master mix reagents kit (PE Applied Biosystems) according to manufacturer's protocol. The real-time RT-PCR reactions were performed in 96-well optical reaction plates in an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems). Total RNA from MUM-2B cells was used to develop the standard curve. MT1-MMP mRNA was normalized to the housekeeping gene *36B4* (23). Samples were run in triplicate and with values reported as a percentage of the control population; statistical analyses to determine significance of the observed differences were performed using the student's *t* test (Sigma Plot for Windows, SPSS Inc.).

Zymography and MT1-MMP Activity Assays. Gelatin zymography was used to examine the levels of MMP-2 activity in MUM-2B, C8161, and C918 cells that were either untreated (DMSO control) or treated with 20 μM LY294002 for 48 h. Serum-free conditioned media was collected and subjected to SDS-PAGE using 0.01% w/v gelatin containing 10% polyacrylamide gel. After electrophoresis, gels were equilibrated in 50 mM Tris-HCl (pH 7.5) with 2.5% Triton X-100 for 30 min at room temperature. Subsequently, the gels were then incubated in 50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, 150 mM NaCl, and 0.02% NaN_3 for 20 h at 37°C. Gels were stained with Coomassie and destained until wash remained clear and cleared zones associated with MMP activity were apparent. The zymogram was digitized and the amount of clearing associated with MMP-2 activity was determined using Scion Image for Windows (β 4.0.2; Scion Corp.), and the values were calculated relative to the control samples normalized to a value of 1.00. To determine MT1-MMP activity, the MT1-MMP activity assay kit (Amersham Biosciences, Piscataway, NJ) was used, following manufacturer's protocol. The activity assay plate was incubated at 37°C for 6.5 h and absorbance read at 405 nm. The experiment was performed in triplicate, and the samples were run in duplicate and values reported as a percentage of the control population; statistical analyses to determine significance of the observed differences were performed using the student's *t* test (Sigma Plot for Windows, SPSS, Inc.).

Results

PI3K Regulates VM *in Vitro*. It has been suggested that PI3K plays a role in kidney tubule formation using kidney epithelial cells (24) and in endothelial cell sprouting (19). To address the role of PI3K during melanoma VM, a specific inhibitor to PI3K was used, LY294002. The highly aggressive uveal MUM-2B (Fig. 1, *A–C*) and C918 (Fig. 1, *G–I*) and cutaneous C8161 (Fig. 1, *D–F*) melanoma cells were cultured on a three-dimensional matrix (consisting of type I collagen) and left either untreated or treated with LY294002 for a period of 4 days, after which time the inhibitor was removed, and the cells were cultured for an additional 4 days to allow for potential recovery. As shown in Fig. 1, *B*, *E*, and *H*, addition of the PI3K inhibitor, LY294002, inhibited VM by the aggressive melanoma cells tested. Furthermore, the effects of PI3K were reversible as the subsequent removal of LY294002 followed by an additional 4 days in culture allowed VM to commence (Fig. 1, *C*, *F*, and *I*). Western blot analyses of whole cell lysates from these experimental samples confirmed a reduction in PI3K activity after addition of LY294002, as determined by using anti-phospho-AKT(Ser^{473}; Fig. 1*J*), a well accepted method for determining PI3K activity (25).

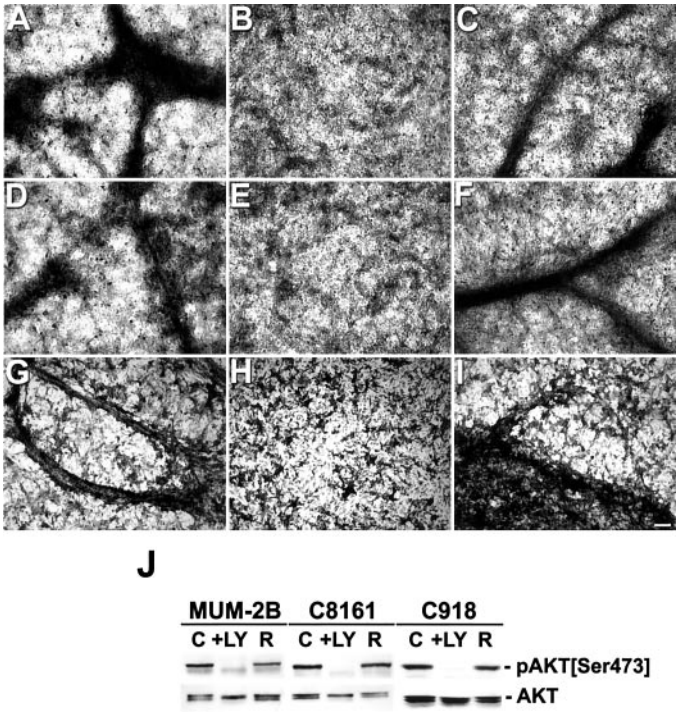

Fig. 1. Addition of a specific inhibitor for PI3K inhibits vasculogenic mimicry in a reversible manner *in vitro*. Brightfield microscopy of the aggressive MUM-2B and *C918* uveal and C8161 cutaneous melanoma cells grown on a three-dimensional matrix in the presence or absence of 20 μM LY294002 and stained by PAS without hematoxylin counterstain. MUM-2B (*A*), C8161 (*D*), and C918 (*G*) aggressive melanoma cells cultured in the absence of LY294002 for 4 days. MUM-2B (*B*), C8161 (*E*), and C918 (*H* and *I*) cultured in the presence of 20 μM LY294002 for 4 days, after which, the LY294002 was removed, and the cells were cultured in fresh medium for an additional 4 days to allow for recovery (*C*, MUM-2B; *F*, C8161, and *I*, C918). *Bar* = 100 μm. *J*, respective whole cell lysates from MUM-2B, C8161, and C918 melanoma cells left untreated (C; DMSO control), treated with 20 μM LY294002 (+LY), or treated and allowed to recover (R) were run on a 10% polyacrylamide gel and probed with phospho-specific anti-AKT antibodies [pAKT(Ser^{473})] stripped and reprobed with anti-AKT antibodies (AKT).

PI3K Affects the Production of Active MMP-2 *in Vitro*. MMP-2 has been implicated in endothelial tubulogenesis (26), melanoma tumor cell invasion (27, 28), and VM (18). Other studies have also implicated PI3K as mediating endothelial cell sprouting through secretion of MMP-2 (19). On the basis of these studies and the current data suggesting that PI3K can regulate both invasion and VM, we sought to determine whether PI3K was capable of regulating MMP-2 in the aggressive MUM-2B, C8161, and C918 melanoma tumor cells. Fig. 2*A* shows that LY294002 treatment led to a 5-fold decrease in the levels of active MMP-2 in the MUM-2B cells and a 3-fold decrease in both the C8161 and C918 cells in a reversible manner. Western blot analyses of representative whole cell lysates confirmed a complete inhibition of PI3K activity after addition of LY294002, as determined by using anti-phospho-AKT(Ser^{473}) antibodies (Fig. 2*B*). On the basis of these results, coupled with our previous observations demonstrating MMP-2 as a necessary component for VM (18), we concluded that PI3K can regulate MMP-2, thus promoting VM *in vitro*.

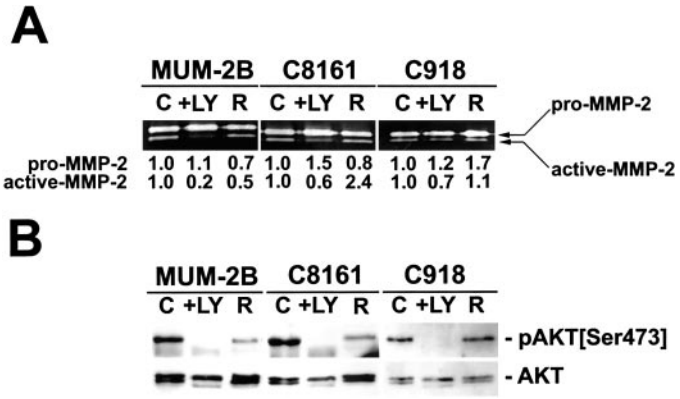

Fig. 2. Effects of LY294002 on MMP-2 activity in MUM-2B, C8161, and C918 melanoma cells. *A*, gelatin zymography of serum-free conditioned media from MUM-2B, C8161, or C918 cells left untreated (C; DMSO control), treated with 20 μM LY294002 (+LY) for 48 h, or treated and allowed to recover (R) for 48 h. Numerical values represent relative density of the pro-MMP-2 and active-MMP-2 bands calculated relative to the control samples and normalized to 1.00. *B*, respective whole cell lysates from MUM-2B, C8161, or C918 melanoma cells left untreated (C; DMSO control), treated with 20 μM LY294002 (+LY), or treated and allowed to recover (R) were run on a 10% polyacrylamide gel and probed with phospho-specific anti-AKT antibodies [pAKT(Ser^{473})] stripped and reprobed with anti-AKT antibodies (AKT).

PI3K Affects the Expression and Activity of MT1-MMP *in Vitro*. MT1-MMP has been shown to cleave pro-MMP-2 into its active conformation. On the basis of gelatin zymography (shown in Fig. 2*A*), we observed that LY294002 inhibited the ability of the aggressive melanoma cells to convert pro-MMP-2 into its active form, and therefore, we sought to determine whether PI3K was acting on MT1-MMP. For these studies, we used both an MT1-MMP activity assay in addition to real-time RT-PCR. The data show that addition of LY294002 decreased both the activity (Fig. 3*A*) and expression (Fig. 3*B*) of MT1-MMP in the MUM-2B, C8161, and C918 melanoma cells in a reversible manner. The aggressive MUM-2B uveal melanoma cells displayed a statistically significant inability to recover both the expression of MT1-MMP and its activity 48 h after treatment with 20 μM LY294002. Allowing the MUM-2B cells to recover for an additional 48 h restored MT1-MMP activity and expression to control values (data not shown). These results coupled with the previous observations suggested that PI3K acts through MT1-MMP to activate MMP-2, thus promoting VM *in vitro*.

PI3K Affects Cleavage of Laminin 5γ2 Chain *in Vitro*. Previous results have suggested that active MMP-2 and MT1-MMP cleave laminin 5γ2 chain to produce γ2′ and γ2x fragments, which are promigratory in nature (29, 30) and necessary for melanoma VM (18). Thus far, our results have indicated that PI3K is able to regulate the activation of MMP-2 through MT1-MMP, and therefore, we sought to investigate the possibility that PI3K could mediate the formation of these laminin associated promigratory fragments. Western blot analysis of whole cell lysates treated with LY294002 revealed a decrease in the levels of both γ2′ and γ2x promigratory fragments in a reversible manner as demonstrated in Fig. 4*A*. These data suggest that PI3K is able to regulate the cleavage of laminin 5γ2 chain through MT1-MMP and MMP-2 and ultimately affect VM *in vitro*. A hypothetical model describing a novel signaling pathway underlying melanoma VM based on the data generated in the current study is presented in Fig. 4*B*.

Discussion

Previous studies from our laboratory proposed important roles for MMP-2, MT1-MMP, and laminin 5γ2 chain in melanoma VM (18). In the current study, we addressed the regulation of MMP-2 and MT1-MMP activity with respect to the cleavage of laminin 5γ2 chain in highly aggressive melanoma tumor cells, which facilitates VM. The data revealed that PI3K is an important mediator of melanoma VM. The experimental evidence suggests that PI3K mediates the expression and activity of MT1-MMP, which in turn mediates the activation of MMP-2. Furthermore, the findings indicate that the activation of MT1-MMP and MMP-2, regulated by PI3K, is necessary for the cleavage of laminin 5γ2 chain to form the γ2′ and γ2x promigratory fragments. These observations extend previously published data implicating MT1-MMP, MMP-2, and laminin 5γ2 chain as key molecular components underlying melanoma VM (18). Taken together, these data suggest that PI3K promotes VM in highly aggressive melanoma cells by regulating the activity of MT1-MMP, MMP-2, and the cleavage of laminin 5γ2 chain—uncovering a novel signaling pathway with therapeutic implications.

Regarding the *in vivo* biological implications of VM, significant new findings have revealed the presence of a fluid-conducting meshwork in xenografts of human cutaneous and uveal melanoma that corresponds to laminin-positive vasculogenic-like networks (14, 15). Using a combination of i.v. tracers, together with routine, confocal, and immunoelectron microscopy, these studies have shown that tracers are found inside traditional, endothelial-lined vasculature, and

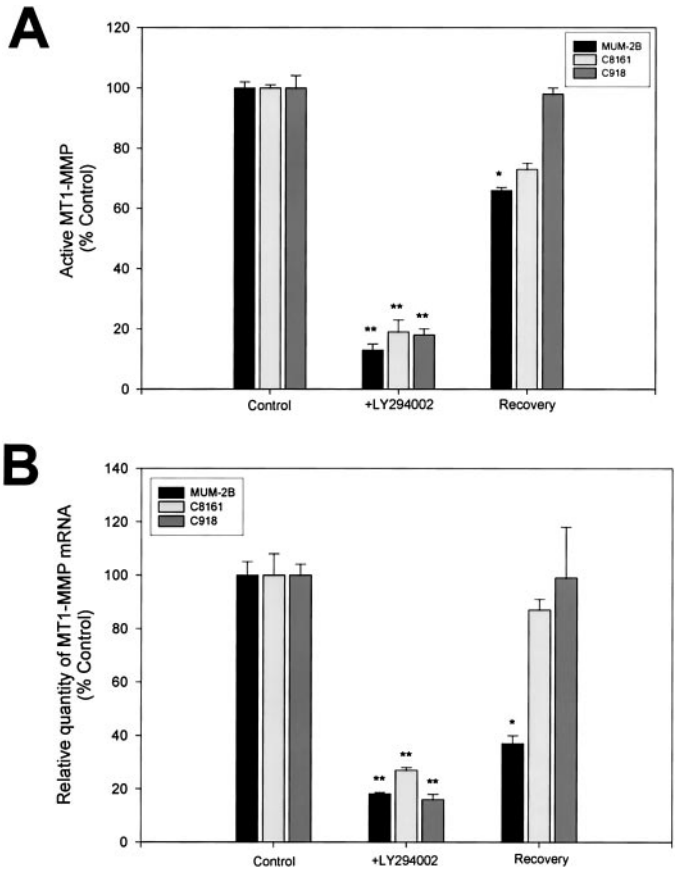

Fig. 3. Measurement of the activity and expression of MT1-MMP in the MUM-2B, C918, and C8161 cells after treatment with LY294002. *A*, MT1-MMP activity measured using an ELISA assay in cells left untreated (Control; DMSO control), treated with 20 μM LY294002 (**, +LY294002 compared with control; MUM-2B, $P < 0.001$; C8161, $P < 0.001$; C918, $P < 0.001$), or treated and allowed to recover (Recovery) for 48 h (* recovery compared with control; MUM-2B, $P < 0.01$). Values represent a percentage of the control population. *B*, real-time RT-PCR indicating the relative quantity of MT1-MMP mRNA from cells cultured on a three-dimensional type I collagen matrix under serum-free conditions and left untreated (Control; DMSO control), treated with 20 μM LY294002 (+LY294002) for 48 h (**, +LY294002 compared with control; MUM-2B, $P < 0.001$; C8161, $P < 0.001$; C918, $P < 0.001$), or treated and allowed to recover (Recovery) for 48 h (* recovery compared with control; MUM-2B, $P < 0.01$). Values represent a percentage of the control population.

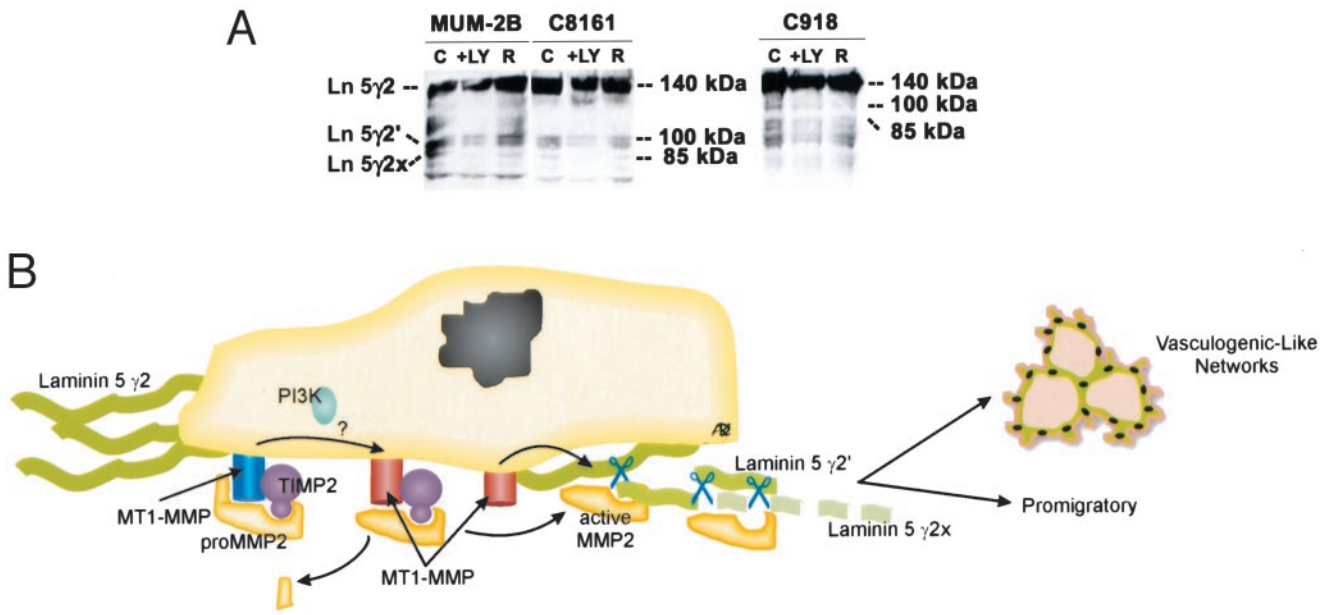

Fig. 4. Analysis of laminin 5γ2 chain produced by MUM-2B, C8161, and C918 cells cultured on a three-dimensional type I collagen matrix after treatment with LY294002. *A*, Western blot analysis of whole cell lysates from untreated (C; DMSO control) and cells treated with 20 μM LY294002 (+LY) for 4 days or cells treated and allowed to recover (R) for 4 days. Lysates were separated on a 7.5% polyacrylamide gel and transferred to nitrocellulose. Blots were probed with antilaminin 5γ2 antibody. *B*, hypothetical model highlighting the potential mechanism(s) by which PI3K regulates melanoma vasculogenic mimicry *in vitro*. In this model, PI3K regulates MT1-MMP activity, which promotes the conversion of pro-MMP into its active conformation through an interaction with TIMP-2. Both enzymatically active MT1-MMP and MMP-2 may then promote the cleavage of the laminin 5γ2 chain into the pro-migratory γ2′ and γ2x fragments. The deposition of these fragments into the tumor extracellular milieu may result in increased migration, invasion, and ultimately vasculogenic mimicry by highly aggressive melanoma tumor cells.

extravenously along channel-like spaces created by laminin-positive patterned loops and networks encasing spheroidal nests of tumor cells (14, 15, 31, 32). This fluid conducting meshwork has been shown to contain plasma (and some RBCs) around the tumor cell-lined spheroidal nests (15) and appears to serve as an extravascular fluid pathway. The functional relevance of this finding is still unclear, but this intratumoral meshwork system may provide a nutritional exchange for aggressive tumors that might prevent early necrosis (reviewed in Ref. 32). Thus, to gain a greater perspective of the molecular underpinnings of VM in melanoma, we focused on key steps involved in the formation of the laminin-rich networks *in vitro* of which MT1-MMP and MMP-2 are essential catalysts.

MMPs have been implicated in a variety of mechanisms that promote tumor progression (33). Specifically, MT1-MMP and MMP-2 are key mediators of invasion, metastasis, tumor angiogenesis, and recently tumor cell VM (18, 34). Numerous studies have indicated that MT1-MMP is important for endothelial tubulogenesis in fibrin gels (26), in endothelial cell migration on three-dimensional collagen gels (35), and both MT1-MMP and MMP-2 are up-regulated when endothelial cells are cultured on a three-dimensional matrix (36). In addition, it was reported that angiopoietin-1 stimulates pro-MMP-2 production in endothelial cells promoting sprouting angiogenesis, which can be blocked by addition of PI3K inhibitors (19), thus linking PI3K activity and MMP-2 activity with angiogenesis. Furthermore, a role for PI3K-mediated MMP activity in malignant gliomas has recently been suggested (37). In this study, Kubiatowski *et al.* (37) established that treatment of C6 glioma cells with LY294002 decreased cellular invasion in conjunction with a decrease in the levels of secreted MMP-9 and MMP-2.

Our data additionally link PI3K activity with MT1-MMP activity and MMP-2 activation by demonstrating that inhibition of PI3K results in decreased expression and activation of MT1-MMP. We hypothesize that the decrease in active MMP-2 is a result of the diminution of MT1-MMP expression and activation. The TIMP-2 mediates the cleavage of pro-MMP-2 into its active conformation by bringing it into close proximity to an active MT1-MMP molecule that then cleaves pro-MMP-2 and releases the active form. Seftor *et al.* (18) previously found that addition of recombinant TIMP-2 only retards melanoma VM *in vitro*, and so the importance of this particular molecule in regulating VM is unclear. On the basis of these previous observations, we did not address the role of TIMP-2 in mediating MT1-MMP activity in the current study. Interestingly, the data revealed slight differences in the effects of the PI3K inhibitor among the three melanoma cell lines tested. The data indicate that treating the cells with the LY294002 inhibitor resulted in a slightly greater reduction in active MT1-MMP in the MUM-2B and C918 cells *versus* the C8161, however, the effects on VM remained similar. Likewise, there was a statistically significant inability to recover both MT1-MMP activity and expression 48 h after treatment with LY294002 in the MUM-2B cells, suggesting that these cells may be slightly more sensitive to the effects of this drug. These differences could be attributed to the different routes of metastatic dissemination associated with the origin of the different cell lines. For example, uveal melanoma cells generally disseminate through purely hematogenous routes (38, 39), whereas cutaneous melanoma cells may disseminate by either hematogenous or lymphatic routes (39, 40).

While Wortmannin is also considered a specific inhibitor of PI3K activity, its use is limited to short-term experiments because of its instability in aqueous solutions and its tendency to bind serum proteins (41). We examined the effects of wortmannin on MT1-MMP activity using a short-term assay and found it inhibited MT1-MMP activity in a manner similar to LY294002 (data not shown). Extrapolation of this short-term effect might suggest that longer exposure to Wortmannin would also lead to the inhibition of PI3K, resulting in decreased MT1-MMP activity.

The mechanism by which PI3K influences MT1-MMP expression or activation remains unknown; therefore, additional experiments are needed to understand this intriguing regulatory pathway. It has been shown that protein kinase B/AKT, a downstream effector of PI3K, is implicated in regulating the expression of

MMP-9 in an nuclear factor-κB-dependent manner and results in increased migration and invasion in HT1080 cells (42). Moreover, it has been demonstrated that sustained exposure to H_2O_2 increases MT1-MMP expression with subsequent activation of pro-MMP-2 in HT1080 cells, a phenomenon that was found to be mediated by PI3K and nuclear factor-κB activation (43). We did not observe any change in the levels of pro- or active-MMP-9 with the addition of PI3K inhibitors in our cell system (data not shown). Furthermore, addition of function blocking anti-MMP-9 antibodies did not affect VM by aggressive melanoma cells *in vitro* (18), and so we have concluded that MMP-9 is not critical for melanoma VM in the aggressive cell lines tested to date. However, it is tempting to speculate that PI3K may regulate the expression of MT1-MMP in the aggressive melanoma tumor cells in a similar manner to its regulation of MMP-9 in HT1080 cells. Recently, Zhang *et al.* (44) identified a role for PI3K/AKT signaling in regulating the expression of MT1-MMP and MMP-2 activity in Lewis lung carcinoma cells treated with type 1 insulin-like growth factor. They found that addition of specific PI3K inhibitors down-regulated the expression of MT1-MMP activated by the insulin-like growth factor-I R receptor (44).

The role of the laminin 5 γ2′ and γ2x promigratory fragments as a potential inducer of VM is quite intriguing. Seftor *et al.* (18) demonstrated that these fragments are deposited into the extracellular milieu by highly aggressive melanoma cells and that they can serve as stimulatory signals for poorly aggressive tumor cells to assume a vasculogenic. It was also demonstrated that treatment of aggressive melanoma cell preconditioned matrix with anti-laminin 5γ2 antibodies blocked those stimulatory signals (18). Other studies have indicated that MT1-MMP and MMP-2 are necessary for the cleavage of laminin 5γ2 chain into the γ2′ and γ2x fragments. Using normal epithelial cells as well as numerous tumor cell types, it was shown that either MT1-MMP alone or in combination with MMP-2 activation, resulted in cleavage of laminin 5γ2 chain resulting in the formation of γ2′ and γ2x fragments, which promoted migration of breast epithelial cells and various tumor cell types (18, 29, 30). In the current study, we found that addition of specific inhibitors to PI3K decreased both MT1-MMP and MMP-2 activity. Furthermore, we found that this decrease in MT1-MMP and MMP-2 activity resulted in a decrease of the γ2′ and γ2x promigratory fragments. On the basis of these results, we speculate that PI3K activity regulates the migration, invasion, and VM potential of highly aggressive melanoma cells by promoting the activation of MT1-MMP, MMP-2, and the cleavage of laminin 5γ2 chain into promigratory fragments, as presented in the hypothetical model in Fig. 4*B*. Furthermore, we hypothesize that addition of LY294002 to three-dimensional cultures of aggressive melanoma cells might block their ability to produce stimulatory signals that could be interpreted by poorly aggressive melanoma cells to take on a more aggressive phenotype; experiments are currently underway to test this presumption.

VM is an example of tumor cell plasticity with myriad implications. Understanding the key molecular events that regulate the formation of vasculogenic-like networks and laminin-rich fluid conducting meshworks by highly aggressive tumor cells may provide additional therapeutic intervention strategies. In this study, we have identified PI3K as a key regulator of the expression and activation of MT1-MMP, activation of MMP-2, cleavage of the laminin 5γ2 chain, and ultimately VM in aggressive melanoma tumor cells. These results provide important clues into the regulatory mechanisms underlying VM and specifically identify PI3K as a potential new target in a novel signaling cascade.

Acknowledgments

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