Molecular Regulation of Tumor Cell Vasculogenic Mimicry by Tyrosine Phosphorylation: Role of Epithelial Cell Kinase (Eck/EphA2)

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Abstract

During embryogenesis, blood vessels are formed initially by the process of vasculogenesis, the in situ differentiation of mesenchymal cells into endothelial cells, which form a primitive, patterned vasculogenic network. This is followed by angiogenesis, the sprouting of new vessels from pre-existing vasculature, to yield a more refined microcirculation. However, we and our collaborators have recently described a process termed “vasculogenic mimicry,” which consists of the formation of patterned, tubular networks by aggressive melanoma tumor cells (in three-dimensional cultures in vitro), that mimics endothelial-formed vasculogenic networks and correlates with poor clinical prognosis in patients. Previous microarray analysis from our laboratory comparing the highly aggressive versus the poorly aggressive melanoma cells revealed a significant increased expression of tyrosine kinases associated with the aggressive melanoma phenotype. Because of the important role of protein tyrosine kinases in phosphorylating various signal transduction proteins that are critical for many cellular processes (e.g., cell adhesion, migration, and invasion), we examined whether protein tyrosine kinases are involved in melanoma vasculogenic mimicry. Immunofluorescence analysis of aggressive melanoma cells forming tubular networks in vitro showed that tyrosine phosphorylation activity colocalized specifically within areas of tubular network formation. A phosphotyrosine profile of the aggressive melanoma cells capable of forming tubular networks indicated differences in tyrosine phosphorylated proteins compared with the poorly aggressive melanoma cells (incapable of forming tubular networks). Most notably, we identified epithelial cell kinase (EphA2) as being one receptor tyrosine kinase expressed and phosphorylated exclusively in the aggressive metastatic melanoma cells. Furthermore, general inhibitors of protein tyrosine kinases hindered tube formation, and transient knockout of EphA2 abrogated the ability of tumor cells to form tubular structures. These results suggest that protein tyrosine kinases, particularly EphA2, are involved in the formation of tubular networks by aggressive melanoma tumor cells in vitro, which may represent a novel therapeutic target for further clinical investigation.

Introduction

During embryogenesis, blood vessels are formed initially by the process of vasculogenesis, the in situ differentiation of mesenchymal cells into endothelial cells, which form a primitive, patterned vasculogenic network. This is followed by angiogenesis, the sprouting of new vessels from pre-existing vasculature, to yield a more refined microcirculation. In tumors, it is widely accepted that this necessary vascularity occurs exclusively via angiogenesis (1). However, there are confounding reports indicating a perplexing lack of correlation between vascular density indices and tumor aggressiveness in a variety of cancers (2, 3). In fact, we and our collaborators have recently documented the lack of classical angiogenesis within the tumor compartment of a subset of human uveal melanomas, which correlated with a poor clinical outcome in patients (4). These aggressive tumors were further characterized by a unique pattern of extracellular matrix-rich networks (surrounding spheroids of tumor cells) with RBC-containing channels in many areas lined exclusively by tumor cells (4). Human melanoma cell lines derived from both aggressive primary and metastatic tumors, but not from poorly aggressive tumors or normal tissues, were also able to recapitulate similar patterned networks and form perfusable tubular structures, surrounding spheroids of tumor cells when cultured on three-dimensional matrices (4). The ability of melanoma tumor cells to mimic endothelial cells and form vasculogenic networks has been termed “vasculogenic mimicry” by us and others (4, 5).

Microarray analysis of the aggressive melanoma cell lines revealed differential expression of various protein tyrosine kinases as compared with the poorly aggressive melanoma cell lines (4, 6). Signaling transduction pathways, consisting of PTKs, which are largely responsible for relaying signals through the cell by way of phosphorylation events, play critical and diverse roles in regulating cell adhesion, migration, and proliferation. The increased expression of various protein tyrosine kinases expressed by the aggressive melanoma cells prompted us to determine whether they may play a role in mediating vasculogenic mimicry.

In this study, we tested the hypothesis that melanoma cells that express a specific profile of tyrosine phosphorylated proteins would be facilitated in their ability to engage in vasculogenic mimicry, as determined by the formation of patterned tubular networks in vitro. Immunofluorescence staining of the aggressive melanoma cells grown on three-dimensional matrices indicated that areas of the cultures containing patterned tubular networks also showed high levels of tyrosine phosphorylation. The addition of general inhibitors of PTKs resulted in the inability of the aggressive melanoma cells to form the tubular networks. Further molecular analysis revealed Eck (Eck/EphA2) as one receptor PTK that was expressed and phosphorylated exclusively in the aggressive metastatic uveal melanoma tumor cells. Transient knockout of EphA2 expression in the aggressive uveal melanoma tumor cells resulted in the inhibition of tubular network formation by these cells. Together, these results demonstrate that...
PTKs, particularly EphA2, play a critical role in the formation of matrix-rich tubular networks by aggressive melanoma cells and may constitute one of the essential molecular determinants of vasculogenic mimicry demonstrated by melanoma.

Materials and Methods

Cell Culture. The MUM-2B and MUM-2C cell lines used in this study were derived from a metastatic uveal melanoma explant within the liver, specifically from the MUM-2 heterogeneous cell strain (7). The MUM-2B cells display an epithelioid phenotype and are highly invasive, whereas the MUM-2C cells display a spindle shape and are poorly invasive, as described previously (7). These cell lines were maintained in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum (Gemini Bioproducts, Calabasas, CA) and 0.1% gentamicin sulfate. Cell cultures were determined to be free of Mycoplasma contamination using the GenProbe rapid detection system (Fisher, tofu, IL).

Three-Dimensional Cultures. Fifteen μl of type I collagen (3.02 mg/ml; Collaborative Biomedical, Bedford, MA) were dropped onto 18-mm no. 1 glass coverslips in 12-well tissue culture plates. Forty-five μl of 100% ethanol were dropped onto each coverslip, and the collagen was allowed to polymerize for 5 min at room temperature. Four-hundred and fifty μl of 1× PBS were dropped onto each coverslip, and the collagen was allowed to polymerize for an additional 5 min at room temperature. Five × 10⁵ tumor cells were then seeded on top of the collagen gel. For experiments with herbinyclic, A, cells were allowed to incubate on the collagen for 3 h, after which the medium was removed and replaced with fresh medium containing herbinyclic A (final concentration, 875 nM; Life Technologies, Inc.). Fresh medium containing herbinyclic A was added to these cultures every 24 h for 4 days. Cells were determined to be viable by use of the trypan blue exclusion test.

Immunoprecipitation, Electrophoresis, and Immunoblotting. Fifty μl of type I collagen (3.02 mg/ml; Collaborative Biomedical) were dropped into each well of a six-well tissue culture plate. One hundred and fifty μl of 100% ethanol were added to each well, and the collagen was allowed to polymerize for 5 min at room temperature. One hundred and fifty μl of 1× PBS were added to each well, and the collagen was allowed to continue polymerizing for an additional 5 min at room temperature. Two × 10⁶ tumor cells were then seeded into each well. At the appropriate time points, cells plus the matrix were removed from the dishes by cell scraping. The medium, matrix, and cells were then centrifuged at 12,500 rpm for 5 min at 4°C. The pellets were washed once with ice-cold 1× PBS and then centrifuged at 12,500 rpm for 5 min at 4°C. The pellets were lysed with 1× RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, and 0.5% deoxycholate) containing 25 μg/ml leupeptin (Sigma Chemical Co., St. Louis, MO), 10 μg/ml aprotinin (Sigma Chemical Co.), 1 mM sodium orthovanadate, and 2 mM EDTA and then centrifugation at 12,500 rpm for 5 min. The protein concentration of the samples was determined using a BCA Protein Assay Reagent kit (Pierce Corp., Rockford, IL), and whole cell lysates were analyzed by 10% SDS-PAGE and stained with Cooamassie BBR-250 (Sigma Chemical Co.) to ensure equal loading (data not shown). Immunoprecipitations were performed by continuous shaking for 1 h at 4°C with 5 μg of primary antibody/reaction, followed by 1-h incubation in the presence of protein A-Sepharose beads (15 μl; Sigma Chemical Co.). The protein A-Sepharose was washed thoroughly with RIPA buffer, and the protein was recovered by boiling in electrophoresis SDS sample buffer. Samples were separated by 10% SDS-PAGE and transferred to nitrocellulose (Schleicher and Schuell, Keene, NH). Blots were blocked overnight at 4°C and for 1 h at room temperature in Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBST), 0.1% BSA, and 2% cold fish gelatin (Sigma Chemical Co.). Blots were incubated with either anti-phosphotyrosine antibody (1:500 PY-20; Transduction Laboratories, San Diego, CA) or anti-EphA2 antibody (1 μg/ml; Upstate Biotechnology, Lake Placid, NY) for 1 h at room temperature with agitation, followed by incubation with a horseradish peroxidase-conjugated antimum secondary antibody (1:5000; The Jackson Laboratory, Bar Harbor, ME). Blots were developed using an enhanced chemiluminescence detection kit (ECL; Amersham Pharmacia Biotech, Piscataway, NJ).

Immunocytochemistry. Coverslips containing three-dimensional cultures were fixed with 3.7% formaldehyde in PBS for 10 min and then stored in TBS (50 mM Tris-HCl, 150 mM NaCl, and 1% sodium azide) at 4°C. Prior to staining, coverslips were rinsed with TBS. Coverslips where dipped in a 0.5% Triton X-100 solution for 6 min, followed by a rinse with TBS for 5 min. Coverslips where incubated for 1 h with anti-phosphotyrosine antibody (1:50 PY-20; Transduction Laboratories) and Texas Red-conjugated phallolidin (1: 50; Molecular Probes, Eugene, OR) at room temperature. After incubation, the coverslips were rinsed with TBS for 5 min. The coverslips were then incubated for 1 h with a FITC-conjugated antimum IgG (1:50; Cappel ICN, Irvine, CA) at room temperature and subsequently rinsed with TBS for 5 min. The coverslips were then mounted on glass slides using permanent mounting media.

For cross-sectional analysis of these three-dimensional cultures, transwells (Fisher Scientific) containing three-dimensional cultures of MUM-2B cells were frozen and sectioned at 7 μm. Slides were aceton fixed for 5 min, rinsed in PBS, and stained with anti-EphA2 kinase antibodies (1:50; Upstate Biotechnology), using LSAB plus alkaline phosphatase kit (Dako, Carpinteria, CA). Sections were then exposed to Vector red chromogen (Vector Laboratories, Burlingame, CA) for up to 5–7 min, rinsed in distilled water, counterstained with methyl green (0.5% aqueous purified with chloroform; Poly Scientific, Bay Shore, NY) for 10 min, and coverslipped with permanent mounting medium.

To help visualize the extracellular matrix-rich tubular networks within the three-dimensional cultures prepared on glass coverslips, the coverslips were stained with PAS, omitting hematoxylin counterstaining to reduce visual noise; black and white photography with a green filter was also used to enhance the PAS-positive patterns. Pictures were taken using an Axioskop 2 (Carl Zeiss, Inc., Thornwood, NY) and Spot camera (Diagnostic Instrument, Inc., Sterling Heights, MI) using the Zeiss Axiosvision 2.0.5 software (Carl Zeiss, Inc.).

Transient Transfections. To knock out the expression of EphA2 in the aggressive MUM-2B uveal melanoma cells, they were transiently transfected with phosphorothioate-modified antisense oligonucleotides (5′-CCAGCAG-TACCGTCTTCTTGCCCTGCGGCCG-3′), inverted antisense oligonucleotides (5′-GCCGTTCCGTCCTTCTACATGACGACC-3′), or untransfected to serve as a vehicle control. The cells were transfected with 8 μg of DNA using Lipofectamine PLUS Reagent (Life Technologies, Inc.), following the manufacturer’s protocol. Twenty-four h after transfection, the cells were harvested and seeded onto three-dimensional cultures consisting of type I collagen. After 48 h, the cultures were analyzed by bright-field microscopy for the presence of tubular networks using an Olympus IX-70 inverted microscope. Images were captured onto 35 mm Kodak T-Max professional film using an Olympus OM-4T camera. At 0 and 24 h, the treated cells were harvested from the three-dimensional matrix, and a Western blot was prepared as described previously (8). The Western blot was probed with anti-EphA2 antibodies and with anti-paxillin antibodies (a generous gift from Keith Burrige, University of North Carolina, Chapel Hill, NC) to serve as an internal loading control.

Results

Our experimental approach focused on the differential ability of aggressive versus poorly aggressive uveal melanoma cells, isolated from the same patient, to engage in vasculogenic mimicry in vitro. The aggressive MUM-2B uveal melanoma cell line is able to form a pattern of hollow tubular networks (demonstrated by PAS stain) when cultured on a three-dimensional matrix composed of type I collagen, as demonstrated in Fig. 1, A, C, and E, on days 1, 3, and 6, respectively. However, the poorly aggressive MUM-2C uveal melanoma cell line is unable to form tubular networks under the same culture conditions, as depicted in Fig. 1, B, D, and F, during days 1, 3, and 6, respectively. Light microscopic analysis of cross-sections of the patterned networks formed by MUM-2B cells demonstrates the hollow lumen-like structures that exist within the tubular networks (Fig. 1E, inset).

PTKs and tyrosine phosphorylation of various signal transduction proteins are important for cellular adhesion, migration, and invasion. Thus, we addressed whether tyrosine phosphorylation was important for the formation of tubular networks by the aggressive melanoma cells. To test this premise, MUM-2B aggressive uveal melanoma cells
were grown on a three-dimensional matrix and dual labeled with an antibody to phosphotyrosine (to reveal tyrosine phosphorylated proteins) and with phalloidin (to demonstrate F-actin filaments). An area of tubular network formation under bright-field microscopy is shown in Fig. 2A, and the corresponding dual-fluorescence image is shown in Fig. 2C, which delineated an area of intense phosphotyrosine staining associated with the tumor formed tubular networks. Actin staining highlights the cellular components contained within the spheroids of tumor cells surrounded by the tubular networks. In contrast, when the poorly aggressive MUM-2C cells were cultured on a three-dimensional matrix, they were unable to form tubular networks, as shown in Fig. 2B; the fluorescence image from the same field (shown in Fig. 2D) revealed dispersed phosphotyrosine staining of the MUM-2C cells, along with a lack of organization into patterned tubular networks as seen with the aggressive MUM-2B cells.

On the basis of the observations shown in Fig. 2, which suggested that tyrosine phosphorylation may be associated with tubular network formation, we next examined whether inhibiting tyrosine kinases could affect tubular network formation by the aggressive MUM-2B uveal melanoma cells. Consequently, MUM-2B cells were cultured on a three-dimensional matrix in the presence or absence of 875 nM herbimycin A. Under these conditions, cells can be cultured up to 96 h without toxic effects (9). After 4 days in vitro, the aggressive MUM-2B uveal melanoma cells cultured in the absence of herbimycin A were able to form tubular networks as expected (Fig. 3A). However, the herbimycin A-treated MUM-2B cells were inhibited in their ability to form complete tubular networks; instead, clusters of cells and extracellular matrix were observed in the absence of organized networks, as demonstrated by PAS histochemistry (Fig. 3B). These cells were viable based on the trypan blue exclusion test (data not shown). Removal of the herbimycin A allowed the MUM-2B cells to recover and resume the formation of tubular networks within 4 additional days (Fig. 3C).

These results suggested an important role for tyrosine kinases and hence tyrosine phosphorylation in tubular network formation.

Fig. 1. Bright-field microscopy of MUM-2B and MUM-2C human melanoma cells cultured on three-dimensional type I collagen matrices stained with PAS without hematoxylin counterstain. A, C, and E, MUM-2B aggressive melanoma cells at day 1 (A), day 3 (B), and day 6 (E). Inset E shows a cross-section of the tubular networks indicating that they contain hollow lumen-like structures. B, D, and F, MUM-2C poorly aggressive melanoma cells at day 1 (B), day 3 (D), and day 6 (F). Bar, 100 μm. Inset, image was viewed using a ×63 oil immersion lens.

Fig. 2. Bright-field and immunofluorescence microscopy showing colocalization of phosphotyrosine and F-actin proteins within areas of tubular network formation in aggressive MUM-2B and in cell clusters of poorly aggressive MUM-2C melanoma cells, cultured on a three-dimensional matrix for 4 days. A and C, MUM-2B three-dimensional cultures demonstrating bright-field image with corresponding fluorescent image (A), dual-labeled for phosphotyrosine proteins (PY-20; FITC) and F-actin (phalloidin; Texas Red); arrows, tubular networks (C). B and D, MUM-2C bright-field (B) and dual-labeled fluorescence image (D). Bar, 100 μm.
EphA2 was expressed exclusively in MUM-2B cells (data not shown), which was confirmed by Western blot analysis of an anti-EphA2 antibodies (Fig. 4A). Furthermore, light microscopic immunohistochemical analysis of three-dimensional cultures of MUM-2B vasculogenic networks showed positive staining with anti-EphA2 antibodies (Fig. 4D), compared with no staining in the control tissues (Fig. 4E). These results suggest that phosphorylation of EphA2 may play a critical role in the aggressive MUM-2B uveal melanoma cells ability to form tubular networks and engage in vasculogenic mimicry.

To directly test the role of EphA2 in the process of tubular network formation, antisense oligonucleotides were used to transiently diminish EphA2 expression in the aggressive uveal melanoma cells. The treated cells were then assessed for their ability to develop tubular structures when cultured on a three-dimensional matrix. Light microscopic analysis of three-dimensional cultures indicated that 48 h after transfection, the cells that were treated with the inverted antisense oligonucleotides were able to form tubular networks as shown in Fig. 5A, whereas the cells treated with the EphA2 antisense oligonucleotides were inhibited in their ability to initiate tubular networks (Fig. 5B). Western blot analysis confirmed that antisense oligonucleotide treatment of aggressive melanoma cells decreased EphA2 expression (Fig. 5C). Levels of paxillin were assessed as relatively unchanged in the control and experimental samples. These results implicate the importance of EphA2 expression in the ability of the aggressive uveal melanoma cell to engage in tubular network formation in vitro.

Discussion

PTKs play important and diverse roles in controlling cell adhesion, migration, and invasion. These kinases can be either transmembrane microarray analysis data (4, 6), suggested that EphA2 could be the candidate kinase involved in this process. EphA2 is a Mr 130,000 protein that can be regulated via tyrosine phosphorylation (9) and has been implicated in the process of angiogenesis (10). Immunoprecipitation with anti-phosphotyrosine antibodies was performed on the same cell lysates, and the Western blot was probed with anti-EphA2 antibodies. The Western blot showed that EphA2 was indeed tyrosine phosphorylated in the MUM-2B cells (Fig. 4B). A Northern blot analysis verified that EphA2 was expressed exclusively in MUM-2B cells (data not shown), which was confirmed by Western blot analysis of an anti-EphA2 immunoprecipitation with these lysates (Fig. 4C).
Epithelial cell kinase is a member of the Eph family of protein receptor tyrosine kinases. Eph is a large family that currently consists of 14 members (10). The EphA2 receptor tyrosine kinase has a molecular weight of Mᵋ 130,000 and contains 976 amino acids (11). The cytoplasmic domain contains the tyrosine catalytic domain of the protein and a putative phosphorylation site at Y-772 (11). EphA2 is normally expressed in tissues that contain a high proportion of epithelial cells such as skin, intestine, lung, ovary, and kidney (11–13). The ligand for EphA2 is ephrin-A1 (otherwise known as B61; Ref. 14, 15). Binding of ephrin-A1 causes EphA2 to become phosphorylated; however, it has been found that EphA2 can also be constitutively phosphorylated in unstimulated cells (12). Early experiments using a yeast two-hybrid system showed that EphA2 was able to bind to the COOH-terminal SH2 domain of the p85 subunit of PI3K, and activation of EphA2 by ephrin-A1 then stimulated activation of PI3K (16). Also, using the same yeast two-hybrid system, a novel protein containing SH3 and SH2 adapter modules was found to bind to activated EphA2 and was named src-like adapter protein (17). Recently, it was revealed that the nonactivated form of EphA2 was able to associate with FAK in vivo, and the complex becomes disrupted, leading to the dephosphorylation of FAK and paxillin upon activation of EphA2 by ephrin-A1 (18). These results demonstrated decreases in integrin-mediated cell adhesion, spreading, and migration (8, 18). Lastly, the dephosphorylation of FAK by activation of EphA2 could be suppressed by addition of inhibitors to protein tyrosine phosphatases, suggesting that activated EphA2 may recruit various phosphatases.

EphA2 is not normally expressed in normal melanocytes but is often up-regulated in various primary melanoma tumors as well as those from metastatic lesions (4, 19–22). The role of EphA2 in tumor progression remains largely unknown. Studies have implicated EphA2 to play a role in the growth and proliferation of EphA2 expressing melanoma cells, as well as induction of vascularization in advanced melanoma (22), thus underscoring its importance in tumor progression.

Our results validated the hypothesis that melanoma cells expressing a specific profile of tyrosine phosphorylated proteins would be facilitated in their ability to participate in vasculogenic mimicry, the formation of patterned tubular networks in vitro, and suggests that protein tyrosine kinases leading to phosphorylation of various signal transduction proteins are also important for the process of vasculogenic mimicry. We have identified one such receptor protein tyrosine kinase, EphA2, as being exclusively expressed and phosphorylated in the highly aggressive uveal melanoma cell line, MUM-2B, but not in the poorly aggressive MUM-2C cell line. These data extend the molecular microarray analysis of these two cell lines, isolated from the same patient, which demonstrated a genetic instability or deregulation in the aggressive cells that allowed them to indiscriminately express genes that are associated with embryonic-like, pluripotent stem cells (4), which may facilitate their ability to engage in vasculogenic mimicry and form patterned tubular networks.

The signaling pathways downstream of EphA2 need to be further examined to discern its precise role in vasculogenic mimicry. Interestingly, it has been reported that kidney epithelial cells expressing elevated levels of PI3K activity are able to form branching tubules when cultured on a three-dimensional matrix composed of type I collagen (23). On the basis of these observations and ours, it is tempting to speculate that activated EphA2 could be signaling through PI3K in the aggressive uveal melanoma cells, thus resulting in the formation of tubular networks in three-dimensional matrices.

Our observations reveal for the first time that epithelial cell kinase is a critical molecular determinant for vasculogenic mimicry in aggressive melanoma cells. Inhibition of tyrosine phosphorylation abrogates the ability of the aggressive melanoma cells to form embryonic-like patterns of vasculogenic networks, which may have future
implications for clinical use in treating uveal melanoma tumors containing patterned, extracellular matrix-rich networks surrounding spheroids of tumor cells. Not only have these patterned networks been found during embryogenesis and in the formation of the primitive microcirculation (24–26), but most recently they have been observed in Drosophila tumors containing mutations in the lats gene (large tumor suppressor gene), a model with no blood flow or endothelial cells (27, 28). Although the function of these networks is not completely understood, it is plausible that they may be important in tumor perfusion, dissemination, and drug resistance. Further investigation of Eck may help to elucidate the molecular regulation of vasculogenic perfusion, dissemination, and drug resistance. Further investigation of tumor suppressor gene), a model with no blood flow or endothelial cells.

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