Up-Regulation of EphB4 in Mesothelioma and Its Biological Significance

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Abstract

Purpose: Mesothelioma is a rare malignancy that is incurable and carries a short survival despite surgery, radiation, or chemotherapy. This study was designed to identify novel targets for diagnostic, prognostic, and therapeutic approaches.

Experimental Design: The expression and functional significance of the receptor tyrosine kinase EphB4 was studied in vitro and in a murine model of mesothelioma.

Results: EphB4 was highly expressed in mesothelioma cell lines and primary tumor tissues but not in normal mesothelium. Knockdown of EphB4 using small interfering RNA and antisense oligodeoxynucleotide showed reduction in cell survival, migration, and invasion. EphB4 knockdown initiated a caspase-8-mediated apoptosis and down-regulation of the antiapoptotic protein bcl-xl. EphB4 knockdown also resulted in reduced phosphorylation of Akt and down-regulation of matrix metalloproteinase-2 transcription. In addition, murine tumor xenograft studies using EphB4 oligodeoxynucleotides showed a marked reduction in tumor growth accompanied by a specific decline in EphB4 protein levels, reduced cell division, apoptosis in tumor tissue, and decreased microvascular density.

Conclusions: EphB4 is expressed in mesothelioma, provides a survival advantage to tumor cells, and is therefore a potential novel therapeutic target.

Mesothelioma is a rare neoplasm that most often arises from the serous surface of the pleural and peritoneal cavity (1). There are three main histologic types of malignant mesothelioma (i.e., epitheloid, mixed or biphasic, and sarcomatoid), with survival being higher with the epitheloid and shorter with the sarcomatoid type (2). There is a strong association with asbestos exposure with ~80% of malignant mesothelioma occurring in individuals who have ingested or inhaled asbestos. The disease has an incidence of 3,000 cases per year in the United States with 2,500 deaths ascribed to it (3). Most patients present with locally advanced tumor that is incurable with surgery, radiation, or chemotherapy. This study was designed to identify novel targets for diagnostic, prognostic, and therapeutic approaches.

The expression and functional significance of the receptor tyrosine kinase EphB4 was studied in vitro and in a murine model of mesothelioma. Knockdown of EphB4 using small interfering RNA and antisense oligodeoxynucleotide showed reduction in cell survival, migration, and invasion. EphB4 knockdown initiated a caspase-8-mediated apoptosis and down-regulation of the antiapoptotic protein bcl-xl. EphB4 knockdown also resulted in reduced phosphorylation of Akt and down-regulation of matrix metalloproteinase-2 transcription. In addition, murine tumor xenograft studies using EphB4 oligodeoxynucleotides showed a marked reduction in tumor growth accompanied by a specific decline in EphB4 protein levels, reduced cell division, apoptosis in tumor tissue, and decreased microvascular density.

Conclusions: EphB4 is expressed in mesothelioma, provides a survival advantage to tumor cells, and is therefore a potential novel therapeutic target.

Combining pemetrexed and cisplatinum compared favorably with a response in 41.3% versus 16.7% response following cisplatinum treatment alone, associated with median duration to progression of 5.7 versus 3.9 months. Although there was an increase in survival to a median of 12 months compared with 9 months for cisplatinum alone, long-term survival remained dismal (9). Thus, new treatment approaches are needed with an emphasis on targeted therapeutics.

Progress has been made in identifying alterations in gene expression in mesothelioma to better understand tumor cell biology and identify novel therapeutic targets. For example, we and others have shown that vascular endothelial growth factor (VEGF) and VEGF-C function as autocrine growth factors for mesothelioma (10, 11). VEGF inhibitors, including neutralizing antibodies and antisense molecules, are currently in clinical investigations (12). Epidermal growth factor receptor (EGFR) and platelet-derived growth factor (PDGF) are also expressed in mesothelioma and could provide growth advantage to tumor cells (13-16). The potential benefit of EGFR and PDGF inhibitors is also the focus of clinical trials (17, 18).

To discover additional molecular therapeutic targets that might be incorporated into a multimodality regimen for the treatment of mesothelioma, we studied the expression and function of the receptor tyrosine kinase (RTK) EphB4 and its cognate ligand, EphrinB2. EphB4 is a member of the largest family of RTKs and plays an important role in a variety of processes during embryonic development, including pattern formation, cell aggregation and migration, segmentation, neural development, angiogenesis, and vascular hierarchical remodeling (19-22). EphrinB2, the transmembrane ligand of EphB4, also participates in vascular remodeling via reverse...
signaling. Targeted knockout of either EphB4 or EphrinB2 in mice shows similar phenotypes with disrupted vessel matura-
tion and early embryonic lethality (20). This receptor-ligand pair is not only important in vasculogenesis in the embryo but also essential for defining the boundary between arterial and venous domains (23), which persists in the adult life (24). Venous endothelial cells express EphB4, whereas arterial endothelial cells express EphrinB2 (20, 23). In addition to their role in embryonic development, recent studies have shown that they are implicated in tumor development (25). Aberrant expression of EphB4 in cancer has been suggested in colon cancer, breast cancer, and endometrial carcinoma (26–30).

In the current study, we first show the expression of various receptors of the EphB family and their ligands in mesothelioma. We then show that EphB4 plays an important role in cell survival, migration, and invasion. EphB4 knockdown leads to caspase-8-mediated (extrinsic pathway) apoptosis and a reduc-
tion in the antiapoptotic protein bcl-xl. Targeting EphB4 in vivo led to a specific reduction in EphB4 protein levels that resulted in a significant inhibition in tumor growth combined with a specific reduction in EphB4 protein levels that resulted in a significant inhibition in tumor growth combined with

**Materials and Methods**

**Cell lines and reagents.** NCi H28, NCi H2052, NCi H2373, and MSTO 211H mesothelioma cell lines, MCF-7 breast cancer cell line, 5637 bladder cancer cell line, and 293T human embryonic kidney cell lines, MSTO 211H mesothelioma cell lines, MCF-7 breast cancer cell line, and 293T human embryonic kidney cell line were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in RPM1 1640 supplemented with 10% heat-inactivated fetacl bovine serum (FBS); Life Technologies, Gaithersburg, MD) and penicillin/streptomycin (Invitrogen, Carlsbad, CA). 211H cells were cultured on plates coated with fibronectin (50 μg/mL; Sigma, St. Louis, MO). Primary mesothelioma cells were obtained from tumor samples. Tumor biopsy specimens fixed in formalin were also obtained from 16 patients with mesothelioma. Normal pleura and peritoneum were obtained from patients undergoing surgery for unrelated reasons. EphB4-specific small interfering RNAs (siRNA; Table 1) and phosphorothioate-modified antisense oligodeoxynucleotides (Table 2) were synthesized at University of Southern California/ Norris Cancer Center Microchemical Core Laboratory. Effective and specific antisense oligodeoxynucleotide (AS-10) and siRNA (siRNA 472) that knockdown EphB4 expression in 293T cells transiently transfected with full-length EphB4 were selected for subsequent study (data not shown). Polyclonal EphrinB2 (P20), EphB1 (Q20), EphB2 (C20), EphB3 (L19), EphB4 (C-16), and EphB6 (N19) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal EphB4 antibodies (M91 and VK138, Vasgene, Inc., Los Angeles) were used in Western blotting and immunoprecipitation. Monoclonal antibody against Ki-67 was obtained from DAKO (Carpinteria, CA), β-actin from Sigma, bcl-2 and bcl-xl from Calbiochem (San Diego, CA), and Mcl-1 from BD PharMingen (San Diego, CA). FITC-labeled lycopercisinin lectin was obtained from Sigma.

**Reverse transcription-PCR.** Total RNA was extracted using RNA STAT-60 (Tel-Test, Inc., Friendswood, TX). First-strand cDNA was synthesized from 5 μg total RNA using SuperScript III (Invitrogen). Primer sequences are as follows: EphB4 forward primer 5’-TGA-

**Table 1. Oligodeoxynucleotides**

<table>
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<th>Name</th>
<th>Sequence</th>
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<tr>
<td>Scrambled oligodeoxynucleotide</td>
<td>5’-TACCTGAAGGTGCAGGCACAC-3’</td>
</tr>
<tr>
<td>EphB4 AS-10</td>
<td>5’-ATGGAGGCGCTGCTAGAAA-3’</td>
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**Table 2. siRNAs**

<table>
<thead>
<tr>
<th>Name</th>
<th>siRNA sequence</th>
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<tr>
<td>GFP</td>
<td>5’-GGCGACCGCCUGUAACAUU-3’</td>
</tr>
<tr>
<td>EphrinB2</td>
<td>5’-GCAGAGAAGUCAGCUAAU-3’</td>
</tr>
<tr>
<td>EphB4 472</td>
<td>5’-GGUGAAGUGUCAAGACCGUGU-3’</td>
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**Preparation of digoxigenin-labeled RNA probes.** EphrinB2 and EphB4 PCR products were cloned using the pGEM-T Easy System (Promega, Madison, WI) according to the manufacturer’s description. Primer sequences were 5’-TCCGTGTTGAAAGTCATTGCTG-3’ (forward) and 5’-CTCTGGGAGCAGTCGTG-3’ (reverse) for EphrinB2 that yielded a 296-bp product and 5’-CTTGGAAGAACCCCTGCTG-3’ (forward) and 5’-AGACGGTGAAAGTCGCTTCG-3’ (reverse) for EphrinB4 that yielded a 297-bp product. The authenticity and insert orientation were confirmed by DNA sequencing. The pGEM-T Easy plasmids containing the PCR product of the human EphrinB2 or EphB4 gene were linearized with SpeI or NcoI. Antisense or sense digoxigenin-labeled RNA probes were transcribed from T7 or SP6 promoters by runoff transcription using a digoxigenin RNA labeling kit (Roche, Indianapolis, IN). RNA probes were quantitated by spot assay as described in the digoxigenin RNA labeling kit instructions.

**In situ hybridization.** In situ hybridization was performed as described previously (31). In brief, cells were cultured in Labtech II four-well chamber slides (Nalge Nunc International, Naperville, IL). Cells were washed in PBS at 37°C and fixed for 30 minutes at 25°C in a solution of 4% (w/v) formaldehyde, 5% (v/v) acetic acid, and 0.9% (w/v) NaCl. Slides were rinsed with PBS and stored in 70% ethanol at 4°C until use. Before in situ hybridization, cells were dehydrated, washed in 100% xylene to remove residual lipid, and then rehydrated. Cells were permeabilized by incubating at 37°C with 0.1% (w/v) pepsin in 0.1 N HCl for 20 minutes and postfixed in 1% formaldehyde for 10 minutes. Prehybridization was done for 30 minutes at 37°C in a solution of 4× SSC containing 50% (v/v) deionized formamide. Slides were hybridized overnight at 42°C with 25 ng antisense or sense RNA probes in 40% deionized formamide, 10% dextran sulfate, 1% Denhardt’s solution, 4× SSC, 10 mmol/L DTT, 1 mg/mL yeast tRNA, and 1 mg/mL denatured and sheared.
salmon sperm DNA in a total volume of 40 μL. Slides were then washed at 37°C as follows: twice each for 15 minutes with 2× SSC, for 15 minutes with 1× SSC, for 15 minutes with 0.5× SSC, and for 30 minutes with 0.2× SSC. Hybridization signal was detected using alkaline phosphatase–conjugated anti-digoxigenin antibodies (Roche, Indianapolis, IN) according to the manufacturer’s instructions. Color development was stopped by two washes in 0.1 mol/L Tris-HCl, 1 mmol/L EDTA (pH 8.0) for 10 minutes. Cells were visualized by counterstaining of nucleic acids with Nuclear Fast Red (Vector Laboratories, Burlingame, CA). Slides were mounted with Immumount (Shandon, Astmoor, United Kingdom).

**Western blot.** Crude cell lysates were prepared by incubation in cell lysis buffer [10 mmol/L Tris (pH 7.5), 1 mmol/L EDTA, 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L DTT, 10% glycerol]. Lysates were cleared by centrifugation at 10,000 × g for 10 minutes. Total protein was determined by Bradford assay (Bio-Rad, Hercules, CA). Samples (20 μg protein) were fractionated on a 4% to 20% Tris-glycine polyacrylamide gel and transferred to polyvinylidene difluoride membrane (Bio-Rad) by electroblotting. Membranes were blocked with 5% nonfat milk before incubation with primary antibody at 4°C overnight. Secondary antibody conjugated with horseradish peroxidase was applied for 1 hour at room temperature. The membranes were developed using the SuperSignal West Femto Maximum sensitivity chemiluminescent substrate (Pierce, Rockford, IL). To ensure equal loading and transfer of protein, membranes were stripped with Restore Western Blot stripping buffer (Pierce) and reprobed with β-actin.

**Immunofluorescence studies.** Immunofluorescence was done as described previously (33). Cells were cultured on Labteck II four-well chamber slides and fixed in 4% paraformaldehyde in PBS (pH 7.4) for 30 minutes. The slides were rinsed twice in PBS and preincubated with blocking buffer (0.2% Triton X-100, 1% bovine serum albumin in PBS) for 20 minutes. The slides were then incubated with antibodies to EphB4 or EphrinB2 (1:100) in blocking buffer at 4°C overnight. After washing thrice, the slides were incubated with the fluorescein-conjugated secondary antibodies (Sigma-Aldrich, St. Louis, MO). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI), washed extensively with PBS, and mounted with Vectashield antifade mounting solution (Vector Laboratories). Images were obtained using an Olympus AX70 fluorescence microscope (Melville, NY) and Spot version 2.2.2 (Diagnostic Instruments, Inc., Sterling Heights, MI) digital imaging system.

**Immunohistochemistry.** Sections (5 μm) were deparaffinized in xylene and rehydrated in graded ethanol. Antigen retrieval was done in 10 mmol/L sodium citrate (pH 6.0) at 95°C for 10 minutes. Endogenous peroxide activity was blocked with 3% hydrogen peroxide. The sections were then incubated with the specific primary antibodies (anti-EphB4, 1:50; anti-EphrinB2, 1:100; Ki-67, 1:100) at 4°C overnight. For negative controls, the primary antibodies were replaced with isotype control IgG. Immunostaining was carried out using the Vector Laboratories ABC kit and color was developed with 3,3′-diaminobenzidine (Vector Laboratories). Sections were counterstained with hematoxylin.

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**Fig. 1.** EphB and EphrinB mRNA expression in mesothelioma. **A,** reverse transcription was done on 5 μg total RNA extracted from cultured cells. RT-PCR shows that mRNA for EphB and EphrinB in all four mesothelioma cell lines, whereas control samples (no reverse transcriptase) show no signal. **B,** in situ hybridization for EphB4 and EphrinB2. H28 mesothelioma cells cultured in chamber slides were hybridized with digoxigenin-labeled probes. Positive signal is seen with antisense probes against EphB4 and EphrinB2, whereas sense probes show no signal.
**Cell viability assay.** Cells were seeded on 48-well plates at a density of $1 \times 10^4$ cells per well in a total volume of 200 µL. Following attachment, cells were treated with various concentrations (0-10 µmol/L) of EphB4 antisense oligodeoxynucleotide (AS-10) or scrambled oligodeoxynucleotide as control. After 3 days, medium was changed and fresh oligodeoxynucleotides were added. Following a further 48-hour incubation, cell viability was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (10). EphB4 siRNA or EphrinB2 siRNA (0-100 nmol/L) were introduced into $1/10^4$ cells per well in 48-well plates using LipofectAMINE 2000 according to the manufacturer’s instructions. Cell viability was assayed by MTT 48 hours following transfection.

**Apoptosis ELISA.** Apoptosis was studied in vitro using the Cell Death Detection ELISA<sup>®</sup> PLUS kit (Roche, Piscataway, NJ) according to the manufacturer’s instructions. Briefly, cells were cultured in 24-well plates to 80% confluence and treated appropriately. After 48 hours, $1 \times 10^4$ cells were incubated in 200 µL lysis buffer. Nuclei were pelleted by centrifugation and the supernatant (20 µL) containing the mononucleosomes or oligonucleosomes was incubated with anti-histone-biotin and anti-DNA-POD in streptavidin-coated 96-well plate for 2 hours at room temperature. Color was developed with ABST and absorbance at 405 nm was read in a microplate reader (Molecular Devices, Sunnyvale, CA). Apoptosis was detected in deparaffinized sections of animal tumors by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay using the In situ Cell Fig. 2. EphB4 and EphrinB2 protein expression in mesothelioma. A, total cell lysate (20 µg) from each of the mesothelioma cell lines was probed sequentially for EphB1, EphB2, EphB3, EphB4, EphB6, and EphrinB2 expression. 293T cells transiently transfected with empty vector or full-length EphB4 were used as negative and positive controls for EphB4 expression, respectively (right). B, signals for EphB4 and EphrinB2 were detected by immunofluorescence in mesothelioma cell lines and primary cell isolate from pleural effusion of a patient with malignant mesothelioma. Specificity of immunofluorescence staining is shown by lack of signal with no primary antibody. Original magnification, ×200. C, immunohistochemical staining of EphB4 and EphrinB2 on mesothelioma biopsy specimen. H&E staining shows the tumor architecture of mesothelioma. Original magnification, ×200. D, protein (20 µg) extracted from fresh normal peritoneum and mesothelioma tissue was used in a Western blot to detect expression of EphB4.
Death Detection kit (Roche, Piscataway, NJ) according to manufacturer’s instructions.

Caspase-8 and caspase-9 activity analysis. Caspase activity was measured by colorimetric assay (R&D Systems, Inc., Minneapolis, MN) according to manufacturer’s instructions. Briefly, cells were transfected with EphB4 siRNA or green fluorescent protein (GFP) siRNA, and after 18 hours, cell lysates were incubated with reaction buffer and the appropriate colorimetric substrate at 37°C for 2 hours. Color development was quantified by measuring absorbance at 405 nm. Routine controls included deletion of cell lysate or substrate.

Wound healing migration assay. Cells were seeded onto six-well plates and cultured until confluent. AS-10 or scrambled oligodeoxynucleotide were added to the medium 24 hours before wounding the monolayer by scraping with a sterile pipette tip. To test the effect of siRNA, confluent cultures were transfected with 50 nmol/L EphB4 siRNA or GFP siRNA 12 hours prior wounding. The repopulation of cell-free zone was examined dynamically and recorded with a Nikon Coolpix 5000 digital camera (Melville, NY) with microscope adapter.

Invasion assay. Cells were transfected with EphB4 siRNA or GFP siRNA using LipofectAMINE 2000, and 6 hours later, 0.5 × 10⁵ cells were transfected into 8 cm Matrigel-precoated inserts (BD Bioscience, Palo Alto, CA). The inserts were placed in companion wells containing RPMI 1640 supplemented with 5% FBS and 5 µg/mL fibronectin as a chemottractant. Following 24-hour incubation, the inserts were removed and the noninvading cells on the upper surface were removed with a cotton swab. The cells on the lower surface of the membrane were fixed in 100% methanol for 15 minutes, air dried, and stained with Giemsa stain for 2 minutes. The cells were counted in five individual high-power fields for each membrane under a light microscope. Assays were done in triplicate for each treatment group.

Phosphorylation analysis. Cells grown in six-well plates were serum starved (0.1% FBS supplemented RPMI 1640 for 24 hours) and treated with 3 µg/mL EphrinB2/Fc (R&D Systems) or Fc fragment alone (The Jackson Laboratory, Bar Harbor, ME) clustered with anti-human Fc (The Jackson Laboratory) for 60 minutes. Cleared cell lysates were incubated with 2 µg/mL EphB4 monoclonal antibody (VK138) overnight at 4°C. Antigen-antibody complexes were immunoprecipitated by the addition of 20 µL protein G-Sepharose (Santa Cruz Biotechnology) in PBS for 1 hour at room temperature. Immunoprecipitates were analyzed by Western blot with phosphotyrosine–specific antibody (clone 4G10, Upstate, Lake Placid, NY) at 1:1,000 dilution. To monitor immunoprecipitation efficiency, a duplicate membrane was probed with EphB4-specific monoclonal antibody (M91).

In vivo tumor growth studies. Male BALB/c nu/nu mice (9 weeks old) were implanted with 5 × 10⁶ cells in the flank. After confirming equal tumor sizes, mice bearing the xenografts were randomly divided into three groups (12 mice per group) and treatment started 5 days after tumor implantation. EphB4 AS-10 or scrambled oligodeoxynucleotide dissolved in sterile physiologic saline (0.9% NaCl) was given i.p. at a dose of 20 mg/kg daily for 3 weeks. The mice were monitored clinically and body weight was measured. Tumor volume was calculated as 0.52 × length × width², where length and width are the largest and smallest extents of the palpable tumor. FITC-labeled lectin (4.5 mg/kg) was infused into animals 30 minutes before sacrifice. Frozen tumor sections were counterstained with DAPI and microvascular density was evaluated using a fluorescent microscope.

Statistical analysis. Mean tumor volumes and number of cells staining positive on immunohistochemistry were compared using Student’s t test, with P < 0.05 considered significant.
EphB4 and EphrinB2 are expressed in mesothelioma cell lines. The expression of different EphB receptors and Ephrin ligands in malignant mesothelioma cell lines was determined at the RNA and protein levels. RT-PCR showed that all of the four mesothelioma cell lines express EphB1, EphB2, EphB3, EphB4, EphB6, EphrinB1, EphrinB2, and EphrinB3 (Fig. 1A). We confirmed the expression of EphB4 and EphrinB2 by in situ hybridization. Representative data are shown for H28 cell line. Specific signal for EphB4 and EphrinB2 transcripts was detected using antisense probes. Sense probes for both served as negative controls (Fig. 1B).

Protein expression was determined by Western blot in all four cell lines. Varying levels of EphB receptors were expressed in the cells (Fig. 2A, left). We further characterized the expression of EphB4 and EphrinB2. A specific band for EphB4 was seen at 120 kDa and EphrinB2 at 37 kDa on Western blot (Fig. 2A, right) in all four mesothelioma cell lines but not in the negative control 293T human embryonic kidney cell line.

Expression of EphB4 and EphrinB2 was confirmed in all the cell lines cultured on chamber slides by immunofluorescence (Fig. 2B).

Evidence of expression of EphB4 and EphrinB2 in clinical samples. Tumor cells isolated from the pleural effusion of a patient with pleural malignant mesothelioma were spun onto slides and stained for EphB4 and EphrinB2. Both EphB4 and EphrinB2 were observed in freshly isolated cells (Fig. 2B, rightmost lane). To determine whether these results were a true reflection of expression in the diseased state, tumor biopsy samples were subjected to immunohistochemical staining for EphB4 and EphrinB2. Twelve of the 16 cases showed positive staining confined to tumor cells (Fig. 2C). We also analyzed protein expression by Western blot analysis on freshly harvested mesothelioma samples. Whereas tumor specimens showed high levels of EphB4 expression, normal peritoneal tissue showed no expression (Fig. 2D).

EphB4 is involved in the cell growth, apoptosis, migration, and invasion of mesothelioma. To define the significance of expression of EphB4 and EphrinB2 on the biological behavior...
of mesothelioma, we first examined their effect on cell growth by knocking down the expression with siRNA. siRNA specific to EphB4 and EphrinB2 were screened in 293T cell line lacking endogenous expression but transiently transfected with expression vectors for EphB4 and EphrinB2. siRNAs that knockdown expression >80% after 48-hour treatment were selected for future use (data not shown). Activity of these active EphB4 and EphrinB2 siRNA was then confirmed in mesothelioma cell lines (data not shown). Transfection of H28 cell line with EphB4 siRNA 472 produced a dose-dependent inhibition of cell viability. Transfection of EphrinB2 siRNA alone did not have the inhibitory effect. Combination of EphB4 and EphrinB2 siRNA did not augment growth inhibition over EphB4 siRNA alone (Fig. 3A, left). The EphB4 siRNA was active in a similar dose-dependent manner in the 211H cell line as well (Fig. 3A, middle) but had no effect on the EphB4-negative Kaposi sarcoma cell line, SLK (Fig. 3A, right).

The involvement of EphB4 in cell survival was further confirmed with EphB4 antisense oligodeoxynucleotides. Incubation of cultured H28 or 211H cells with EphB4 antisense (AS-10) generated the same dose-dependent inhibition of cell growth (Fig. 3B, left and middle, respectively). AS-10 had no effect on survival of SLK cells (Fig. 3B, right). These results indicate that EphB4 plays a critical role in mesothelioma cell survival.

Apoptosis as a result of EphB4 knockdown was tested by measuring levels of cytoplasmic nucleosomes. A dose-dependent increase in apoptosis was observed when H28 cells (Fig. 4A, left) or 211H cells (Fig. 4A, right) were transfected with EphB4 siRNA

Fig. 5. EphB4 regulates cell migration and invasion. A, H28 cells (left) and 211H cells (right) were seeded on six-well plates and cultured until 90% confluent. EphB4 siRNA or GFP siRNA (50 nmol/L each) was transfected into cells with LipofectAMINE 2000. The cell layer was scraped and photographed immediately and after 24 hours. Photomicrographs of representative >20 fields. B, a similar assay was done on H28 cells (left) and 211H cells (right) treated with AS-10 or sense oligodeoxynucleotide (10 μmol/L each). Representative photomicrographs were taken immediately and 24 hours after wounding. C, Matrigel invasion assay. Invasion of H28 cells (top) or 211H cells (bottom) into Matrigel was studied in a modified Boyden chamber assay. Cells were transfected with EphB4 siRNA or GFP siRNA (50 nmol/L each). Cells migrating to the underside of the Matrigel-coated insert in response to 5 μg/mL fibronectin in the lower chamber were fixed and stained with Giemsa. Representative photomicrographs. Cell numbers were counted in five individual high-powered fields. Columns, mean; bars, SE.
RT-PCR.

The successful treatment of mesothelioma to improve survival may depend on the identification of novel targets. In the present study, we found that EphB4 is expressed in all three subtypes of mesothelioma (epithelioid, H28 and H2052; sarcomatoid, H2373; and biphasic, 211H). Each cell line showed a different level of EphB4 expression, with the highest level observed in epithelioid type cell line H28. The expression of EphB4 in mesothelioma was confirmed in clinical samples. Due to the small number of cases, we are unable to analyze the relationship between the expression of EphB4 and tumor subtype, grade, and stage. However, studies have shown that overexpression of EphB4 is inversely related to prognosis in endometrial carcinoma, squamous cell carcinoma of the head and neck, and breast cancer (29, 34–36). Further investigation in a large prospective study is required to determine the importance of EphB4 as a marker of progression and metastasis for mesothelioma.

We have shown that EphB4 expressed in mesothelioma cells can be activated by stimulation with EphrinB2, and in tumors, EphB4 stimulation may be similarly achieved by the coexpression of its ligand, EphrinB2. Similar coexpression of this receptor-ligand pair has been shown in other tumor types, including endometrial, lung, and colon carcinoma (30, 31, 37).
On the other hand, a ligand-independent mechanism is also conceivable, relying on activation as a consequence of overexpression. Overexpression leading to constitutive activation has been observed with EphB2 (38). To determine the possible biological role of EphB4 in mesothelioma, we examined changes in cell viability by targeted disruption of EphB4 expression using antisense and siRNA in two different mesothelioma cells (211H and H28). Down-regulation of EphB4 led to a dose-dependent inhibition in cell viability. The arrest in cell growth was mediated by an increase in apoptosis. The up-regulation of caspase-8 activity and relatively unchanged caspase-9 activity indicate that apoptosis occurred through activation of the extrinsic pathway. We also found that bcl-xl levels fell with EphB4 knockdown, whereas no effect was observed in the levels of bcl-2 and Mcl-1. Similar to our results, it has been reported previously that bcl-xl is expressed at high levels in mesothelioma, whereas the expression of bcl-2 and Mcl-1 is very low (39). Reduction in bcl-xl as observed in our studies may thus have significant effect in mesothelioma cell viability. Further work is needed to define the role of bcl-xl, if any, in EphB4-mediated cell survival signal in mesothelioma.
EphB4 activation allows interaction with a variety of cytoplasmic signaling proteins. Many of these proteins have been implicated in regulating cell morphology, cytoskeletal organization, attachment, and motility (40–42). Activation of EphB4 leads to downstream signaling via PI3K; in agreement with which, we show a decline in the activation status of the downstream molecule Akt following EphB4 knockdown. To our knowledge, this is the first report that shows a role for EphB4 in regulation of Akt with a potential role in tumor cell survival. It has been shown that EphB4 receptor signaling mediates endothelial cell migration (40). EphB4/NeuT double transgenic animals develop metastatic tumors in contrast to NeuT transgenic animals, which form tumors only in the mammary gland without metastases (43). We found that the inhibition of EphB4 significantly inhibits migration and invasion. MMPs play a significant role in tumor invasion and angiogenesis. MMP-2 is overexpressed in mesothelioma (44). In a recent study, it has been shown that MMP-2 and MMP-9 are activated and function in endothelial migration on activation of EphB4 (40). Our findings that EphB4 regulates MMP-2 but not MMP-9 in mesothelioma may suggest a potential role for EphB4 in regulation of tumour migration and invasion. The inhibition of EphB4, apart from directly inhibiting tumor survival, may thus prevent tumor progression as well as metastasis.

To further elucidate the possible therapeutic role of targeting EphB4 in the treatment of mesothelioma, we conducted in vivo efficacy studies. EphB4-specific oligodeoxynucleotide significantly inhibited tumor growth, reduced tumor cell proliferation, and induced apoptosis in comparison with control or scrambled oligodeoxynucleotide treatments. Tumor growth inhibition can result from a direct effect on tumor cells as observed in vitro. In addition, EphB4 on tumor cells bind EphrinB2 on tumor vessels and thereby induce an angiogenic response (45). Down-regulation of EphB4 may thus have an additional and independent effect on tumor vasculature as seen in our study. Hence, EphB4 knockdown has a dual inhibitory effect on tumor growth by directly inhibiting tumor cell survival as well as inhibiting tumor-associated angiogenesis.

In conclusion, we show the expression of the RTK EphB4 in malignant mesothelioma. Receptor overexpression may favor uncontrolled cell growth, migration, and tumor progression. EphB4 targeting could serve as novel therapy for patients with mesothelioma.

References

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