Eph-B2/ephrin-B2 interaction plays a major role in the adhesion and proliferation of Waldenstrom’s Macroglobulinemia

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ABSTRACT

Purpose: The Ephrin-receptors (Eph) are found in a wide range of cancers and correlate with metastasis. In this study, we characterized the role of Eph-B2 receptor in the interaction of Waldenstrom’s Macroglobulinemia (WM) cells with the bone-marrow-microenvironment.

Experimental Design: We screened the activity of different RTKs in WM patients and found that Eph-B2 was over expression compared to control. Also, we tested the expression of Ephrin-B2 ligand on endothelial-cells and bone-marrow-stromal-cells isolate from WM patients. We then tested the role of Eph-B2/Ephrin-B2 interaction in the adhesion of WM cells to endothelial-cells and bone-marrow-stromal-cells; the cell-signaling induced by the co-culture in both the WM cells and the endothelial cells; WM cell proliferation, apoptosis and cell cycle in vitro and tumor progression in vivo; and in angiogenesis.

Results: Eph-B2-receptor was found to be activated in WM patients compared to control, with a 5-fold increase in CD19+ WM cells, and activated cell-adhesion signaling, including FAK, Src, p130, paxilin and cofilin, but decreased WM cell chemotaxis. Ephrin-B2 ligand was highly expressed on endothelial-cells and bone-marrow stromal-cells isolated from WM patients and on HUVEC cells, and induced signaling in the endothelial cells promoting adhesion and angiogenesis. Blocking of ephrin-B2 or Eph-B2 inhibited adhesion, cytoskeletal signaling, proliferation, and cell cycle in WM cells which was induced by co-culture with endothelial cells, and decreased WM tumor progression in vivo.

Conclusion: Ephrin-B2/Eph-B2 axis regulates adhesion, proliferation, cell cycle and tumor progression in vivo through the interaction of WM with the cells in the BM microenvironment.
STATEMENT OF TRANSLATIONAL RELEVANCE.

Ephrin-receptors are receptor tyrosine kinases that regulate adhesion, motility and metastasis in many malignancies. In this study, we examine the role of Ephrin B2 receptor/Ephrin ligand interaction in Waldenstrom’s Macroglobulinemia (WM) in the context of the bone marrow microenvironment. We found that Eph-B2 receptor was overexpressed in primary WM cells. The inhibition of Eph-B2 in WM cells and/or inhibition ephrin-B2 on endothelial cells decreased the adhesion of WM cells to endothelial cells, and consequently, decreased proliferation, cell-cycle progression, signaling, and tumor progression in WM cells. These studies delineate the role of Eph-B2/ephrin-B2 in the regulation of tumor cells interaction with endothelial cells and bone marrow stromal cells in the bone marrow. The development of agents that target Eph-B2/Ephrin-B2 interaction can prevent tumor dissemination, adhesion, and proliferation within the context of the bone marrow microenvironment.
INTRODUCTION

Receptor tyrosine kinases (RTKs) are high affinity cell surface receptors for many polypeptide growth factors, cytokines and hormones (1). RTKs are key regulators of normal cellular processes and in the development and progression of many types of cancer (2). The binding of growth factor to the extracellular domain of RTKs activates the receptor and induces a cell signaling cascade that promotes cell survival and proliferation (1, 2).

Ephrin receptors (Eph) represent the largest family of RTKs, including 16 members divided into two classes; Eph-A and Eph-B. The classification is based on the affinity of the receptor to different ligands, Ephrin-A and Ephrin-B (3-5). A-type Ephrins are tethered to the cell membrane by a glycosylphosphatidylinositol anchor, while B-type receptors have a trans-membrane domain that is followed by a short cytoplasmic region (3).

While used extensively throughout embryogenesis and development, Eph receptors are rarely detected in adult tissues. Eph receptors and Ephrin ligand serve as a guidance system to position cells and modulate cell morphology, especially in embryogenesis (6, 7). In adult organisms, Eph/Ephrin interactions can also trigger a wide array of cellular responses, including cell boundary formation, motility, adhesion and egress (8, 9). Elevated levels of expression of both Eph A and B receptors were found in a wide range of cancers including melanoma, breast, prostate, pancreatic, gastric, and esophageal and colon cancer (10). Moreover, increased Eph expression correlates with more malignant and metastatic tumors, consistent with a role of Ephrin in governing cell movement (11).

Waldenstrom’s Macroglobulinemia (WM) is a low grade non-Hodgkin lymphoma, characterized by the presence of abnormal lymphoplasmacytic cells producing high levels
of IgM macroglobulins (12). WM is characterized by widespread involvement of the bone
marrow, which provides a protective environment for the survival and proliferation of these
cells (13). The specific tropism of these cells to the bone marrow niches indicates their
dependence on adhesion and critical interaction with the bone marrow microenvironment
(14). Inhibiting the adhesion of WM cells to the BM microenvironment reduces survival
and proliferation of the tumor cells(15). Direct cell-cell interaction or stimulation through
cytokines secreted by endothelial cells has been shown to regulate tumor proliferation in
multiple myeloma (16-18). However, the survival effect of these interactions is poorly
understood in WM.

In this study, we aimed to characterize the role of RTKs in the interaction of WM
cells with the BM microenvironment. We focused on the role of Eph-B2 receptor, which
was found to be over-expressed in WM cells, and its interaction with Ephrin in the BM
microenvironment including on endothelial cells and BM stromal cells (BMSCs).
Moreover, we tested the effect of inhibiting the Eph/Ephrin interaction in WM cells and
endothelial cells through loss of function experiments and blocking antibodies. We showed
that Eph/Ephrin interaction regulates cell adhesion, proliferation, and cell-cycle progression
and tumor progression in WM.
MATERIALS AND METHODS

Reagents

Recombinant ephrin-B2, recombinant SDF1α, Human phosoho-RTK Array Kit and anti-Ephrin-B2 antibody were purchased from R&D (Minneapolis, MN). Anti-Eph-B2 antibody was purchased from Santa-Cruz Biotechnology, Inc. (Santa Cruz, CA). All monoclonal antibodies for western blotting were purchased from Cell Signaling Technologies (Danvers, MA). siRNA for the knock down of Eph-B2 was obtained from Dermacon (Lafayette, CO, USA).

Cells

BCWM1, an IgM secreting lymphoplasmacytic cell line obtained from a patient with WM, and other IgM-secreting cell lines (MEC-1, RL) were used in this study. The BCWM1 was a kind gift from Dr. Treon (Dana-Farber Cancer Institute, Boston, MA). MEC-1 was a kind gift from Dr. Neil Kay (Mayo Clinic, Rochester, MN). RL was purchased from the American Tissue Culture Collection (Manassas, VA). All cell lines were cultured in RPMI-1640 containing 10% fetal bovine serum (Sigma Chemical, St Louis, MO), 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (GIBCO, Grand Island, NY). The umbilical vein endothelial cell (HUVEC) line were purchased from Cambrex (Walkersville, MD), and cultured in EGM-2 MV media (Cambrex) reconstituted according to the manufacturer’s instructions.
WM patient samples were obtained after approval from the Dana-Farber Cancer Institute Institutional Review Board. Informed consent was obtained from all patients in accordance with the Declaration of Helsinki. Primary WM cells were obtained from BM samples using CD19+ micro-bead selection (Miltenyi Biotec, Auburn, CA) with more than 90% purity as confirmed by flow cytometric analysis.

For isolation of BMSCs, BM specimens were obtained from patients with WM; mononuclear cells were separated by Ficoll-Hypaque density sedimentation and were used to establish long-term BMSCs cultures. Mononuclear cells were suspended in 10 mL of growth medium containing DMEM, 20% fetal calf serum (FCS), Pen (100 U/mL), and Strep (100 pg/mL) in 25-cm² flasks. Cells were incubated at 37°C, for 3-4 weeks, and when an adherent cell monolayer had developed with predominantly fibroblast morphology, cells were harvested using trypsin/EDTA, and used as BMSCs.

**Expression of phosphor-RTKs-Antibody arrays**

Forty-two RTKs were screened using phosphor-RTK array kit following the manufacturer’s recommendations (R&D, Minneapolis, MN). Briefly, cell lysates were diluted with the assay diluents and applied to capture antibodies against the different RTKs spotted in duplicate on nitrocellulose membranes. After binding the extracellular domain of both phosphorylated and unphosphorylated RTKs, unbound material was washed, and a pan anti-phospho-tyrosine antibody conjugated to horseradish peroxidase (HRP) was then used to detect phosphorylated tyrosine on activated receptors by chemiluminescence. Quantification of signal intensity was performed with Image-J software (NIH, Bethesda, MD).
Immunohistochemistry

The expression of Eph-B2 was detected in specimens from BM aspirates from 6 normal subjects and 6 WM patients. Specimens were rinsed with PBS, fixed with 4% formaldehyde in PBS, dehydrated with ethanol, embedded in paraffin blocks, and sectioned. Sections were stained with rabbit anti-human Eph-B2.

Immunoblotting

To test the effect of ephrin-B2 on cytoskeletal signaling in WM, BCWM cells (5x10^6) were serum-starved for 3 hours and then stimulated with ephrin-B2 (100 ng/ml) for 0, 5, 10, 15, 30 and 60 minutes. In addition, cells were activated with different concentrations of ephrin-B2 (0, 50, 100, 250, 500 and 1000 ng/ml) for 15 min.

We tested the effect of treating BCWM.1 cells with anti Eph-B2 (5 μg/ml, 1 hour for cytoskeletal signaling or 12 hours for cell cycle signaling). HUVEC cells were treated with anti-ephrin-B2 (5 μg/ml, 1 hour for cytoskeletal signaling or 12 hours for cell cycle signaling) or the combination of both anti-Eph B2 and anti-ephrin-B2. In the experiments involving co-culture, HUVEC cells were cultured for 24 hours before the experiment in 6-well plates at 5x10^4 cells/well, washed with PBS and treated with the anti-ephrin-B2, and WM cells were then applied for 1 hour. After co-culture WM cells were separated from HUVEC cells using gentle pipetting. Non-treated WM cells cultured with or without HUVEC cells served as control for WM cells in the co-culture, similarly non-treated HUVECs were used as control for HUVECs in the co-culture.
BCWM1 or HUVECs were washed with ice-cold PBS, lysed, and protein concentration was normalized. Proteins were blotted using 8-12% acrylamide gels, transferred to a nitrocellulose membrane; membranes were blocked with 5%-nonfat dry milk in TBS/T buffer and incubated with primary antibodies for p-FAK, p-Src, p-Paxilin, p-p130, p-cofilin, p-Akt, cyclin D3, Cyclin E, p-Rb or α-tubulin overnight at 4°C. The membranes were then washed, incubated with appropriate HRP-conjugated secondary antibody, washed, and developed using luminol base assay. Luminescence was measured using x-ray films.

**Immuno-precipitation**

BCWM1 cells were cultured in the presence of increasing concentrations (0, 50, 100, 500, 1000 ng/ml) of ephrin-B2 in for 15 minutes, lysed, and lysates were pre-cleared by incubating with protein G-agarose beads slurry for 30 minutes at 4°C, and beads were removed by centrifugation. Supernatant were incubated with anti-Eph-B2 antibody with gentle rocking overnight at 4°C. Protein G agarose beads slurry was added and incubated for 3 hours with gentle rocking for 1–3 hours at 4°C. Beads were then collected by centrifugation, washed, and resuspended in 20 μl 3X SDS. The samples were heated to 95–100°C for 5 minutes and samples were then loaded to SDS-PAGE gel (8%) and immunoblotted as described above with pan-anti-p-Tyrosine antibody.

**Adhesion to ephrin-B2 and fibronectin coated plates**

Non-tissue culture 96 well plates were incubated over night at 4°C with 50 μL/well of increasing concentrations (0, 50, 100, 500, 1000 ng/ml) of Ephrin-B2. Then wells were
washed with PBS, blocked with BSA (2 μg/ml) for 1 hour at room temperature and washed
with PBS. BCWM1, MEC1, RL or CD19+ cells were starved for 3 hours, labeled with
Calcein-AM (1 μg/ml), washed and added to the plates (10^5 cells/well) for 1 hour at 37°C.
Non-adherent cells were then washed, and adherent cells were detected by measuring
fluorescence intensity in the wells using a fluorometer (Ex/Em=485/520 nm).

To test the role of ephrin-B2/Eph-B2 in adhesion to fibronectin, WM cells were
stained with Calcein-AM, washed, treated with increasing doses of ephrin-B2 (0, 50, 100,
500, 1000 nM) for 15 min, and applied on a fibronectin coated plates (10^5 cells/well) for
1hr at 37°C. Non-adherent cells were then washed, and adherent cells were detected by
measuring fluorescence intensity in the wells using a fluorometer (Ex/Em=485/520 nm).

Trans-well migration assay

BCWM1 cells were starved 3 hours before migration, applied to the upper chamber
of an 8-micron-pore filters for trans-well migration assay (Costar, NY), and left to migrate
in the absence or presence of increasing concentrations (0, 50, 100, 500, 1000 ng/ml) of
ephrin-B2, in presence or absence of 30 nM SDF-1 in the lower chamber for 4 hours at
37°C. Cells that migrated to the lower chambers were counted using flow cytometry.

Actin polymerization

BCWM1 cells were treated with increasing concentrations of ephrin-B2, fixed in
2% formaldehyde for 15min at room temperature, permeabilized with 0.2% saponin, and
stained with (5μg/ml) phalloidin tagged with either Alexa-Fuor-488. Cells were analyzed
by flow cytometry, or were spun onto slides, mounted, and analyzed by confocal microscopy.

**Expression of ephrin-B2 on endothelial cells and BMSCs.**

Negative fractions of BM aspirates from WM patients and HUVEC cells were treated with mouse anti-human ephrine-B2 antibody or isotype control, followed by a secondary FITC-goat-anti-mouse antibody. BM aspirates cells were then treated with PE-anti-CD31 antibody to gate the endothelial cells population. The expression of ephrin-B2 was measured by flow cytometry as fluorescence intensity of FITC. BM stromal cells were trypsinized, washed, and stained with mouse anti-human ephrin-B2 antibody or isotype control, followed by a secondary FITC-goat-anti-mouse antibody.

**The role of ephrin-B2 and Eph-B2 in adhesion of WM cells to endothelial cells and BMSCs**

A confluent monolayer of HUVECs or BMSC was generated by plating $1 \times 10^4$ cells/well in 96-well plates overnight. The next day HUVECs or BMSCs were treated with 0 or 5 μg/ml anti-ephrin-B2 for 1 hour. BCWM1 or MEC1 cells ($1 \times 10^6$ cells/ml) were serum-starved for 3 hours, pre-labeled with Calcein-AM, treated with anti-Eph-B2 (5 ug/ml) or with ephrin-B2 (100 ng/ml) and then added to the HUVECs or BMSCs. Cells were co-cultured for 1 hour at 37°C, and non-adherent cells were washed. Adherent cells were detected by measuring the fluorescence intensity in the wells using a fluorometer (Ex/Em=485/520 nm).
Proliferation assays with or without endothelial cells

The effect of ephrin-B2 on the proliferation of WM cells was tested by treating BCWM cells with increasing concentrations of ephrin-B2 for 24 hours, and cell proliferation was detected using $^3$H-thymidine uptake or BRDU assay as previously described (19). To test the role of ephrin-B2 and Eph-B2 on WM cells and HUVEC cells proliferation when cultured alone or with endothelial cells, a confluent monolayer of HUVEC cells in 96-well plate wells was obtained by seeding $1 \times 10^4$ cells/well overnight. The next day, BCWM cells ($3 \times 10^3$) were cultured with or without HUVEC cells for 24 hours. In some cases HUVEC cells were treated with anti-ephrin-B2, BCWM cells were treated with anti-Eph-B2 or the combination of both.

Cell cycle analysis

WM cells were fixed with 70% ethanol, washed, RNA was degraded by RNAase, DNA was stained with 5 μg/mL propidium iodide (Sigma-Aldrich, St. Louis, MO), and cells were analyzed by flow cytometry. For studies using co-culture of WM cells with HUVEC cells, a confluent monolayer of HUVEC cells in 24-well plate wells was obtained by seeding $5 \times 10^4$ cells/well overnight. The next day, BCWM cells ($1 \times 10^6$) were cultured with or without HUVEC cells for 24 hours. In some experiments, HUVEC cells were treated with anti-ephrin-B2, BCWM cells were treated with anti-Eph-B2 or the combination of both.

Knockdown of EPH-B2 in BCWM1 cells
BCWM1 cells were cultured in OPTI-MEM media overnight in 6-well-plates, washed, and a mixture of lipofectamine-2000 (5 μl) with scramble-siRNA or anti-EPH-B2 siRNA (100 pmol) in a final volume of 2 ml of OPTI-MEM was added to each well. Transfection solution was replaced with complete media after 24hrs and cells were used after 48 hrs.

Quantitative reverse transcription polymerase chain reaction
Quantitative reverse transcription polymerase chain reaction (qRT-PCR) for expression of Eph-B2 was performed on an Applied Biosystems AB7500 Real Time PCR system as described previously (20). All PCR reactions were run in triplicate and mRNA expression was expressed relative to GAPDH.”

The role of Eph-B2 in tumor progression in vivo.
BCWM1 cells transfected with scramble siRNA or Eph-B2 siRNA were injected to SCID/bg mice (age 7-9 weeks) (n=3 per group); 3x10⁶ cells/ mouse, by IV tail-vein injection. To determine early differences in tumor growth patterns and to confirm that the cells did not loose siRNA inhibition, the mice were sacrificed after 1 week (21, 22). The BM from two femurs of each mouse was extracted. Red blood cells were lysed, and the mononuclear cells were used for Flow Cytometry analysis. Cells were resuspended in blocking solution (2% BSA in PBS) for 30 min on ice, cells were then incubated with mouse-anti-human-CD19 antibody conjugated to Alexa-Fluor-488 (5 μg/ml), and rabbit anti-Eph-B2 followed by incubation in Alexa-633-conjugate of secondary anti-rabbit
antibody. Cells were then washed twice with blocking solution, and the CD19+ cells were analyzed for the expression of Alexa-633-Eph-B2 using BD Canto II flow cytometer.

**Angiogenesis assay**

The role of Eph-B2/ephrin-B2 interaction in the angiogenesis was determined using an In Vitro Angiogenesis Assay Kit (Chemicon, Temecula, CA). HUVECs were cultured in the presence or absence of BCWM1 cells, and presence or absence of anti-EpH-B2 and anti-Ephrin blocking antibodies on polymerized matrix gel at 37°C. After 8 hours, and tube formation by endothelial cells was evaluated using Nikon inverted TE2000 microscope, and a 20x Plan-fluor DIC NA0.5 objective. Images were processed with Adobe Photoshop 7.0 software (Adobe, San Jose, CA). Tube formation was calculated as the total length of the tubes per frame and normalized to non-treated HUVECs. Moreover, the number of WM cells adherent to the tubes was calculated, divided by the total length of tubes per frame, and normalized to non-treated co-culture of BCWM1 cells and HUVECs.

**Statistical analysis**

Results were reported as the mean +/- standard deviation for experiments done in three replicates samples and were compared by the Student t test. Results were considered significantly different for p values less than 0.05.
RESULTS

Eph-B2 is highly expressed in WM cells

We first examined the expression and phosphorylation of different RTKs in primary WM samples and cell lines using an antibody-based RTK-array. As shown in Figure 1A and B, there was activation of RTKs in CD19+ cells from healthy donors and from WM patient samples. Eph-B2 was phosphorylated in WM cell lines, BCWM.1, and IgM secreting cell lines RL and MEC-1. Figure 1A shows quantification of the expression of Eph-B2 in the different groups, demonstrating that Eph-B2 receptor was activated in all patient samples and cell lines compared to control, with a 5-fold increase in CD19+ primary WM cells. Figure 1B, shows representative images of the membranes. These results were confirmed by IHC detection on primary normal subjects and WM patient BM biopsies which showed expression of Eph-B2 in WM cells (Figure 1C). The RTK array included other members of the Eph family including Eph-A1, Eph-A2, Eph-A3, Eph-A4, Eph-A6, Eph-A7, Eph-B1, Eph-B2, Eph-B4 and Eph-B6. The only member that was highly expressed on WM cells and BCWM1 cell line compared to normal control was Eph-B2.

Ephrin-B2/Eph-B2 regulate adhesion
We next examined the activity of Ephrin-B2/Eph-B2 on adhesion WM cells. To test the effect of ephrin-B2 on adhesion, we coated adhesion plates with increasing concentrations of ephrin-B2 and tested the adhesion of starved WM cell lines and IgM secreting cell lines to these adhesion plates. We found that ephrin-B2 increased adhesion of WM cell lines (Figure 1D) and primary cells (Figure 1E), with a maximal increase of 150-170% compared to control in plates coated with 100 ng/ml of ephrin-B2. Furthermore, the treatment of starved WM cells with recombinant ephrin-B2 (the ligand of Eph-B2) activated cell-adhesion signaling. Activation of WM cells with ephrin-B2 100 ng/ml showed that peak activation of Eph and downstream signaling was achieved at 15 minutes. This was evidenced by increased phosphorylation of focal adhesion kinase (FAK) and Src (Figure 1F). We further examined different concentrations of ephrin-B2 on activation of adhesion related proteins at the 15 minute time point and showed that ephrin-B2 induced maximum activation at 100 ng/ml, evidenced by increased phosphorylation of Eph-B2, FAK, Src, p130, paxilin and cofilin, without changing the total Eph-B2 (Figure 1G).

When we tested the effect of activation with ephrin-B2 on adhesion to fibronectin, we found that activation of WM cells with increasing concentrations of ephrin-B2 (0, 50, 100, 500, 1000 nM) did not change the adhesion of WM cells to Fibronectin (Figure 1H).”

**Ephrin-B2/Eph-B2 regulate migration of WM cells**

We next determined the functional effects of Eph-B2 on migration of WM cells. First we tested the effect of ephrin-B2 on the chemotaxis of BCWM1 cells in response to SDF-1. Figure 2A shows that ephrin-B2 did not change the chemotactic response of
BCWM1 cells to SDF-1. However, Figure 2B shows that increasing concentrations of ephrin-B2 significantly decreased spontaneous chemotaxis of WM cells. This is in agreement with the decrease in phosphorylation of myosin-light-chain (pMLC) which is critical for cell movement (Figure 2C). In addition we have tested actin polymerization in BCWM cells after treatment with increasing concentration of ephrin-B2. Figure-2D shows that Ephrin–B2 significantly decreased actin polymerization as quantified by flow cytometry analysis. Figure 2E shows a confirmation of the decrease of actin polymerization in response to ephrin-B2 using confocal imaging.”

**Ephrin-B2 and Eph-B2 regulate adhesion of WM cell to endothelial cells and BMSCs.**

Prior studies have shown that ephrin-B2 is highly expressed on endothelial cells (23). In addition, angiogenesis and the interaction of WM cells with endothelial cells in the bone marrow is critical for tumor proliferation (14, 24). We therefore sought to examine the interaction of endothelial cells and WM cells and the role of ephrin-B2/Eph-B2 in this interaction. We first tested the expression of ephrin-B2 on endothelial cells and BMSCs from bone marrow aspirates of WM patient. We found that ephrin-B2 was expressed on endothelial cells and BMSCs in the BM of WM patients, and also on the endothelial cell line (HUVEC) (Figure 3A).

We further confirmed that the interaction between ephrin-B2 on the endothelial cells and the Eph-B2 receptor on WM cells is critical for cell-cell adhesion. Figures 3B shows that inhibition of Eph-B2 on WM cells or inhibition of ephrin-B2 on endothelial cells reduced the adhesion of WM cells to endothelial cells. Pre-treatment of WM cells
with exogenous recombinant ephrib-B2 compensated the adhesion inhibition induced by blocking the endogenous ephrin-B2 on endothelial cells.

These results were further confirmed by testing the role of ephrin-B2 and Eph-B2 in the induction of cytoskeletal signaling and cell-adhesion related proteins in WM cells by interaction with endothelial cells. Figure 3C shows that the co-culture of WM cells with endothelial cells activates cell-adhesion related proteins including phosphorylation of FAK and Src. Inhibition of either ephrin-B2 on endothelial cells or Eph-B2 on WM cells or both reduced the activation of cell-adhesion pathways in WM cells.

Similar result were obtained when BCWM1 were tested, indicating that the interaction between ephrin-B2 on the BMSCs and the Eph-B2 receptor on BCWM1 cells is critical for cell-cell adhesion (Figure 3D).

**Ephrin-B2 and Eph-B2 induce proliferation of WM cell through the interaction with endothelial cells**

When WM cells were cultured alone, inhibition of Eph-B2 with a blocking antibody induced a mild reduction of WM cell proliferation. The use of anti-ephrin-B2 antibody did not alter the proliferation of WM cells, and did not change the effect of the anti-Eph-B2 antibody (Figure 4A). However, co-culture of WM cells with endothelial cells induced an increase in WM cells proliferation compared to WM cells cultured alone. This effect was abolished by blocking-antibody for the Eph-B2 receptor on WM cells, and significantly decreased by a blocking-antibody for the ephrin-B2 ligand on endothelial cells (Figure 4B).

To investigate the proliferative effect induced by ephrin-B2/Eph-B2 axis in WM cells when cultured with or without endothelial cells, we tested its role on cell cycle of WM
cells. Treatment of WM cells with anti-Eph-B2 or with anti-ephrin-B2 or the combination did not alter the cell cycle regulation of these cells (Figure 4C). Co-culture of endothelial cells with starved WM cells induced transition of cells from G1 to S and G2/M phases, an effect which was reversed with either inhibition of Eph-B2 on the WM cells or inhibition of ephrin-B2 on the endothelial cells by blocking antibodies (Figure 4D). These results were confirmed by immunoblotting showing activation of AKT, Cyclin D3, Cyclin E and pRb in WM cells co-cultured with endothelial cells compared to WM cells alone (Figure 4E). These effects were reversed by inhibition of Eph-B2, ephrin-B2 or the combination of both.

Eph-B2 regulates adhesion and proliferation of WM cells in co-culture with endothelial cells and BMSCs, and decreases tumor progression in vivo.

To confirm the selective role of Eph-B2, we knocked-down the expression of the receptor using siRNA. Figure 5A shows that the expression of Eph-B2 gene was down-regulated to 27% compared to transfection with scramble siRNA. Similarly, the down regulation of Eph-B2 protein expression was confirmed using flow cytometry (Figure 5B). Loss of function of Eph-B2 induced a significant reduction of WM cell adhesion to endothelial cells (Figure 5C) and BMSCs (Figure 5D). Moreover, knockdown of Eph-B2 in WM cells prevented the proliferative effect (Figure 5E) and cell-cycle regulation (Figure 5F) in WM cells induced by co-culture with endothelial cells. Mechanistically, these results were confirmed by immuno-blotting showing that co-culture with endothelial cells increased proliferative pathways (such as AKT), and cell cycle proteins (such as cyclin-D, cyclin-E and pRb), however, these effects were not observed in WM cell that were transfected with Eph-B2 siRNA (Figure 5G).
Moreover, the down regulation of Eph-B2 in WM cell decreased tumor progression in vivo (Figure 5H). One week after injecting 3x10^6 BCWM1 cells to SCID mice, the BM contained 25% of CD19+ cells in mice injected with BCWM1 cells transfected with scramble siRNA, while significant tumor progression was observed in mice injected with BCWM1 cells transfected with Eph-B2 siRNA, with only 10% of CD19+ cells in the BM. Downregulation of Eph-B2 expression in the recovered CD19+ cells was confirmed by flow cytometry Figure 5I.

**WM cells stimulate signaling through ephrin-B2 in endothelial cells and increases angiogenesis.**

We investigated the role of ephrin-B2 receptor on endothelial cells and their regulation of the tumor clone. We investigated the effect of ephrin-B2 and Eph-B2 on regulation of tube formation and angiogenesis induced by WM cells. We found that co-culture of WM cells with endothelial cells induces vessel formation which was decreased by either inhibition of Eph-B2 on WM cell or by inhibition of ephrin-B2 on endothelial cells (Figures 6A and B). Similarly, the adhesion of WM cells to the tubes formed was also decreased by inhibition of either Eph-B2 or ephrin-B2 (Figure 6C). Mechanistically, it was shown that the interaction between WM cells and endothelial cell induced signaling in the endothelial cells through phosphorylation of ephrin-B2 and activation of adhesion related signaling including P130, FAK and Src (Figure 6D). In addition we investigated the effect of blocking ephrin-B2 and Eph-B2 on regulation of the proliferation of endothelial cells...
without co-culture with WM cells. Figure-1E shows no effect on the proliferation of HUVEC cells.

**DISCUSSION**

Eph receptors and Ephrin ligands are both membrane bound, therefore, binding and activation of Eph and Ephrin induces cell–cell interactions (3). The Eph system also affects integrin-mediated cell communication with the extracellular environment (25). Ephrin-B2 can interact with other members of Eph receptors, including EphB1, EphB2, EphB3, EphB4, and EphA4 (26, 27). Activation of Eph receptors and Ephrins has been shown to affect cell attachment by means of integrin and focal adhesion protein-dependent mechanisms (28). Prior studies have shown that Ephrin-A1 induces cell adhesion and actin cytoskeletal changes in fibroblasts in a focal adhesion kinase (FAK)-dependent and p130cas-dependent manner through activation of EphA2 receptor (29).

Eph receptors and Ephrins are overexpressed in many human cancers and correlate with aggressive, invasive and metastatic potential of these cancers. In addition, signaling through Eph/Ephrins can lead to cell adhesion or cell repulsion, depending on which signaling pathways are activated. The role of Eph/Ephrins in low-grade B cell lymphomas has not been previously investigated.

In this study, we used Waldenstrom’s Macroglobulinemia as a model of low-grade B cell lymphoma that has the potential of dissemination and metastasis to the bone marrow
and lymph nodes. We first demonstrated that among all tyrosine kinases, Eph-B2 was highly expressed in primary WM samples and cell lines. The expression of Eph-B2 has been shown in many other cancers such as colon cancer and correlated with aggressive behavior (30). Previous reports have shown that Ephrin-B2 is a key regulator of this process and thereby controls angiogenic, lymphangiogenic and tumorogenic growth (23, 31); therefore, we tested the role of ephrin-B2 and Eph-B2 on the WM interaction with the BM microenvironment, especially the endothelial cells and BMSCs. The interaction of Eph-B2 with ephrin-B2 itself led to WM cell adhesion. We next showed that, when activated with recombinant ephrine-B2, Eph-B2 signaling leads to activation of adhesion and cytoskeletal reorganization molecules including FAK, Src, cofillin and paxillin. These results, therefore, indicate that Ephrin-B2/Eph-B2 interaction in WM regulates cell-cell, which is integrin-dependent in our model system through downstream activation of FAK and Src. These results are in agreement with previous studies showing that Ephrin-induced cytoskeletal re-organization requires FAK and p130 (29). In agreement with previous reports showing that Eph receptors and Ephrins regulate cell migration and tissue assembly (27), we found that ephrin-B2/Eph-B2 interaction down regulated spontaneous migration of WM cells but has no effect on SDF1 induced migration.

We further examined cell-cell interaction by examining the interaction of WM cells with endothelial cells. Previous studies in multiple myeloma and WM have shown that endothelial cells and angiogenesis are critical regulators of tumor proliferation and survival in these tumors of the bone marrow. However, the mechanism by which cell-cell interaction of these cells with endothelial cells regulates cell adhesion and survival has not been previously elucidated. Here, we showed that endothelial cells obtained form patients
with WM expressed ephrin-B2 and the interaction of WM cells (expressing Eph-B2) with endothelial cells led to significant adhesion and proliferation of the tumor cells. Moreover, we have shown that this interaction induced signaling in endothelial cells and activated adhesion-pathways and tube formation. Previous studies have shown that endogenous Eph-B2 and Eph-B4 signaling are required for the formation of capillary-like vascular structures and that Eph-B2 and Eph-B4 activation induced signaling in endothelial cells (32). Other studies have shown that these receptors are also critical for the interaction of multiple myeloma cells with mesenchymal cells. Eph-B4-Fc treatment of myelomatous SCID-hu mice inhibited myeloma growth, osteoclast activation and angiogenesis, and stimulated osteoblastogenesis and bone formation, whereas ephrin-B2-Fc stimulated angiogenesis, osteoblastogenesis, and bone formation but had no effect on osteoclastogenesis and myeloma growth (33). The study in MM did not examine interaction of ephrin-B2 with Eph-B2 on tumor cells. We now demonstrate that ephrin-B2/Eph-B2 is dysregulated in WM cells and is critical for cell-cell interaction of tumor cells with endothelial cells for adhesion, survival and proliferation of WM cells.

We demonstrated that activation of the Eph-B2 receptor with ephrin-B2 did not affect WM cell proliferation and cell cycle; however, it induced activation of adhesion cascades which increased adhesion of WM cells to endothelial cell, which in turn, promoted WM cell proliferation through cell cycle transition. And we showed that down regulation of Eph-B2 in WM cells reduced tumor progression in vivo. The link between adhesion-regulating molecules and proliferative signaling pathways in WM is interesting and may lead to discovery of new therapeutic targets, further investigation in this area is warranted. Here, we showed that these receptors/ligands are critical in the regulation of
adhesion, cell cycle progression and tumor proliferation in WM cells, as well as reverse signaling in the endothelial cells and activating adhesion and tube formation (Figure 7).

In summary, this study examines the interaction of Eph-B2 receptor in a low-grade B cell lymphoma model, Waldenstrom’s Macroglobulinemia, and shows that ephrin-B2/Eph-B2 axis regulates adhesion, activation of downstream signaling of integrin-related molecules, survival, cell cycle and proliferation; and tumor progression in vivo through the interaction of tumor cells with the cells in the BM microenvironment, specifically, by altering adhesion and migration properties. This interaction can be abrogated by specific ephrin-B2 or Eph-B2 inhibitors. Further studies to examine the role of Eph-B2 as a novel therapeutic target in WM and in other B cell malignancies are warranted.
ACKNOWLEDGEMENTS

We would like to acknowledge the contribution of Jennifer Stedman in editing this paper.

AUTHOR CONTRIBUTION

FA and AKA: Performed research, designed research, analyzed data and wrote the paper

PM, TC and RDC Performed Research and analyzed data

YL, PQ, JR, AMR, AS, HTN, YZ and BM analyzed data.

IMG: Designed research and wrote the paper.
REFERENCES


FIGURE LEGENDS

Figure 1: Eph-B2 is highly expressed in WM cells and induces adhesion related cell signaling.

(A) Quantification of the expression of p-Eph-B2 expression in CD19+ cells from healthy donors, WM patients and in BCWM1, MEC1 and RL cell lines. P<0.01 between WM cells from patients or IgM secreting cell lines and normal control. (B) Representative images of phospho-RTK arrays in CD19+ cells from BM of five healthy donors, five WM patients, and in BCWM1, MEC1 and RL. The box highlights p-Eph-B2 expression in these RTK arrays. (C) The expression of EphB2 in specimens from BM aspirates from 6 normal subjects and 6 WM patients detected by immunohistochemistry, showing higher expression of EphB2 in WM samples (Images X40, inserts X100). (D) The adhesion of WM cell lines and IgM secreting cell lines (MEC1 and RL) and primary CD19+ cells (E) to plates coated with increasing concentration of recombinant ephrin-B2 (50-1000ng/mL), p value of <0.01 between control and cells treated with 50ng/ml, and p value <0.05 between control and cells treated with 100-500ng/ml. (F) Immunoblotting for pFAK and pSRC using ephrin-B2 100ng/ml at different time points (0-60min). (G)
Immunoprecipitation for tyrosine phosphorylation on EphB2 showing activation at 15 minutes with different concentrations of ephrin-B2 (0-1000ng/ml). Immunoblotting for Activation of cytoskeletal signaling in BCWM1 cells (pFAK, pSRC, p-P130, p-cofilin and p-Paxillin) after stimulation with increasing concentrations of recombinant ephrin-B2 for 15min. (H) The effect of recombinant ephrin-B2 (50-1000ng/mL) on adhesion to fibronectin.

**Figure 2: The role of Eph-B2/ephrin-B2 axis in regulation of the chemotaxis of WM cells**

(A) The effect recombinant ephrin-B2 (50-1000ng/mL) on SDF1-induced chemotaxis of BCWM1 cells (B) The effect of recombinant ephrin-B2 (50-1000ng/mL) on chemotaxis of BCWM1 cells using an 8-micron-pore filters for transwell migration assay. * indicates significant p value <0.01 compared to control. (C) Immunoblotting for pMLC (cell-motility related signaling) showing decrease in phosphorylation in BCWM1 cells after stimulation with increasing concentrations of recombinant ephrin-B2 (50-1000ng/ml) for 15min. The effect of increasing concentrations of recombinant ephrin-B2 (50-1000ng/ml) for 15min on actin polymerization in BCWM1 cells analyzed by flow cytometry (D) and by confocal microscopy (E).

**Figure 3: The role of Eph-B2/ephrin-B2 axis in regulation of the adhesion of WM cells to endothelial cells and BMSCs.**

(A) Expression of ephrin-B2 on endothelial cells (CD31+), BMSCs isolated from BM aspirates of WM patients and on HUVECs detected by flow cytometry. Ephrin B2 was highly expressed on CD31+ endothelial cells from the bone marrow of WM patients and
HUVEC cells as well as on BMSCs. (B) The effect of the inhibition of Eph-B2 in BCWM1 cells by blocking antibody and/or, inhibition of ephrin-B2, and/or addition of recombinant ephrin-B2 on their adhesion to HUVECs (* p< 0.01 compared to “Non-treated control”). (C) Similar studies as in B in MEC-1 cells. (D) The effect of the inhibition of Eph-B2 in BCWM1 cells by blocking antibody and/or, inhibition of ephrin-B2, and/or addition of recombinant ephrin-B2 on their adhesion to BMSCs (* p< 0.01 compared to “Non-treated control”) (E) The effect of the inhibition of Eph-B2 in BCWM1 cells and/or inhibition of ephrin-B2 in HUVECs by blocking antibody on the cytoskeletal signaling of BCWM1 using immunoblotting for pFAK and pSRC.

**Figure 4: The role of Eph-B2/ephrin-B2 axis in regulation of the progression and cell-cycle effects in WM cells induced by endothelial cells.**

(A) Testing the effect of blocking antibodies against Eph-B2 and ephrin-B2 on the proliferation of BCWM1 and MEC1 cells when cultured alone shows that blocking of Eph-B2 induced mild decrease in cells survival, and no effect was observed by the blocking antibody against ephrin-B2. * p< 0.01 . (B) Proliferation assay using co-culture of WM cells with endothelial cells. The inhibition of Eph-B2 in BCWM1 and MEC1 cells and/or inhibition of ephrin-B2 in HUVECs by blocking antibody inhibited the proliferation of BCWM1 and MEC1 induced by co-culture with endothelial cells. * p< 0.01 and # P< 0.05 compared to “control”. (C) Cell cycle analysis of BCWM.1 cells; neither the antibody against Eph-B2 nor ephrin-B2 had any effect of the cell cycle of BCWM1 cells when cultured alone. (D) Cell cycle analysis of BCWM.1 with or without HUVEC cells; the inhibition of Eph-B2 in BCWM1 cells and/or inhibition of ephrin-B2 in endothelial cells...
inhibited the decrease of G1-phase and increase of S-phase induced in BCWM1 cells induced by co-culture with endothelial cells. (E) These finding were confirmed by immunoblotting, in which the co-culture of WM cells with endothelial cells increased the activity of proliferative signaling (pAKT) and G1 to S-phase related proteins (Cyclin-D, Cyclin-E, pRB). Inhibition of Eph-B2 in BCWM1 cells and/or inhibition of ephrin-B2 in endothelial cells reversed the proliferative and cell cycle effects induced by the endothelial cells.

**Figure 5: The role of Eph-B2 in progression and cell-cycle effects in WM cells induced by endothelial cells.**

(A) RT-PCR for Eph-B2 in BCWM cells after transfection with scramble or Eph-B2 siRNA. (B) The expression of Eph-B2 in BCWM cells after transfection with scramble or anti-EPH-B2 siRNA detected by flow cytometry. The effect of Eph-B2 knockdown in BCWM1 cells adhesion to HUVEC cells (C) and BMSCs (D), showing significant reduction in adhesion with loss of function of Eph-B2, p value <0.01. (E) The effect of Eph-B2 knockdown on proliferation in the presence or absence of HUVEC cells. The co-culture of WM cell with endothelial cells induced proliferation of WM cells, through an increased cell cycle activity. Knocking down Eph-B2 in WM cells reversed the proliferative and cell cycle activity induced by stimulation with endothelial cells. (F) The effect of Eph-B2 knockdown on cell cycle progression in BCWM.1 in the presence or absence of HUVEC cells. (G) Immunoblotting for pAkt and cell cycle regulators from G1 to S-phase related proteins (Cyclin-D, Cyclin-E, pRB). WM cells (scrambled or Eph-B2 knockdown cells) were cultured with or without HUVEC cells. Lysates were used for
immunoblotting. (H) The effect of Eph-B2 knockdown on WM tumor progression in vivo was detected by the percent of CD19+ cells in the BM. (p value =0.01). (I) Confirmation of downregulation of Eph-B2 in the CD19+ cells recovered from the BM.

**Figure 6: WM cells stimulate angiogenesis and induce signaling through activation of ephrin-B2 in endothelial cells.**

(A) Representative imaging showing that co-culture of WM cells with HUVECs induced tube formation (angiogenesis). Inhibition of the interaction between ephrin-B2/Eph-B2 by anti-Eph-B2 or anti-ephrin-B2 reduced tube formation and angiogenesis. (B) Quantification of tube formation described in (A), normalized to tube formation produced by HUVEC alone. (C) Quantification of the number of BCWM1 cells adhered to tubes formed by endothelial cells. (D) Immunoblotting for pEphrin-B2, pP130, pFAK and pSRC in HUVEC cells co-cultured with or without BCWM1 and in the presence or absence of anti-Ephrin-B2 or anti-EPH-B2. (E) The effect of anti-ephrin-B2 and anti-Eph-B2 on the proliferation of HUVECs.

**Figure 7: Hypothesized mechanism of the role of EPH-B2 in the interaction of WM cells with Endothelial cells.**

The hypothesized mechanism of the role of Eph-B2/ehphrin-B2 in promoting cell adhesion, cell proliferation and cell cycle of WM cells through interaction with endothelial cells. The interaction of ephrin B2 from endothelial cells with the Eph-B2 receptor on WM cells leads to activation of cell adhesion signaling including p-paxilin pP130 leading to
activation of pFAK, pSRC, and p-cofilin and increased adhesion to endothelial cells. This ligand-receptor activation also leads to induction of proliferation through activation of Akt and cell cycle progression through G1 to S phase transition. In addition, this interaction will lead to signaling in the endothelial cells through phosphorylation of the ligand ephrin-B2 and induce adhesion signaling, including pP130, pFAK and pSrc.
Figure 1

A

P<0.01

Relative Expression of phospho-EPH-B2

Healthy Donors  WM Patients  BCWM1  MEC1  RL

B

Healthy Donors  WM Patients  Cell lines

BCWM1  MEC1  RL
Figure 1

C

[Image of cellular images labeled NS-1 to WM-6 with magnifications X100 and X20]
Figure 1

D

![Bar graph showing adherent cells as a percentage of control for different Ephrin-B2 concentrations (ng/ml) for BSA, 50, 100, 500, and 1000.](image)

E

![Bar graph showing adherent cells as a percentage of control for different Ephrin-B2 concentrations (ng/ml) for BSA, 50, 100, 500, and 1000.](image)

F

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G

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Figure 2

A

Migrating cells (% of Control)

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SDF 30nM

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C

Ephrin-B2 ng/ml

0 50 100 500 1000

p-MLC

Tubulin

D

Actin Polymmerization (% of Control)

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E

Ephrin-B2 (ng/ml)

0 50 100 500 1000

Images showing different concentrations of Ephrin-B2.
Figure 3

A

Isotype control  Anti-ephrin-B2

CD31 cell Isolated from BM Aspirates of WM patients

WM-1  WM-2  WM-3  WM-4  WM-5  HUVEC

Counts

BM stromal cells Isolated from BM Aspirates of WM patients

WM-1  WM-2  WM-3  WM-4  WM-5

FITC

B

Adhesion to HUVEC (% of Scramble)

BCWM1  MEC1

CTR  Anti EPH-B2 on WM  Anti Ephrin-B2 on HUVEC  Anti Ephrin-B2 on HUVEC + Ephrin-B2 on WM

C

BCWM1 Co-culture HUVEC

Tubulin

D

Adhesion to Stroma (% of Scramble)

CTR  Anti EPH-B2 on WM  Anti Ephrin-B2 on HUVEC  Anti Ephrin-B2 on HUVEC + Ephrin-B2 on WM
Figure 4

A

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Proliferation (% of control)

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Co-Culture WM + HUVEC

C

0% serum

- G1: 65%
- S: 28%
- G2/M: 7%

10% serum

- G1: 46%
- S: 44%
- G2/M: 10%

10% serum + Anti-EPH

- G1: 50%
- S: 39%
- G2/M: 11%

10% serum + Anti-Ephrin

- G1: 48%
- S: 44%
- G2/M: 8%

D

BCWM1 alone

- G1: 63%
- S: 34%
- G2/M: 4%

Anti-EPH

- G1: 65%
- S: 33%
- G2/M: 4%

Anti-Ephrin

- G1: 63%
- S: 30%
- G2/M: 5%

Combo

- G1: 64%
- S: 31%
- G2/M: 5%

E

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Counts
Figure 5

A. Eph-B2 Gene Expression (% of Scramble)

B. Adhesion to HUVEC (% of Scramble)

C. Eph-B2 siRNA (P<0.01)

D. Adhesion to Stroma (% of Scramble)

E. Proliferation (% of Control)

F. Cell Cycle Analysis

G. Co-Culture with HUVEC

H. Tumor Progression (% of CD19+ in the BM)

I. Expression of Eph-B2

P<0.01

Isotype control Anti-EPH-B2

Scramble siRNA

Eph-B2 siRNA

P<0.01

pAKT

Cyclin D3

Cyclin E

pRb

β-Actin

Scramble – alone

Scramble + HUVECs

Scr siRNA

siRNA-Eph-B2

G1: 65%
S: 31%
G2/M: 4%
P<0.05

G1: 42%
S: 50%
G2/M: 8%
P<0.01

G1: 63%
S: 30%
G2/M: 7%
P<0.01

G1: 63%
P<0.01

S: 32%
G2/M: 5%
P<0.01
Figure 6

A

HUVEC alone

HUVEC + BCWM1 Co-Culture

HUVEC + BCWM1 Co-Culture + anti-Eph-B2

HUVEC + BCWM1 Co-Culture + anti-ephrin-B2

HUVEC + 10nM VEGF (Positive Control)
Figure 6

B

Tube formation (% of HUVEC alone)

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Co-culture with BCWM1 - + + +

Anti-EPH-B2 - - + -

Anti-Ephrin-B2 - - - +

p-Ephrin-B2

p-P130

α-Tubulin

p-SRC

p-FAK

α-Tubulin

C

Adhesion of WM cell to Tubes (% of control)

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Ephrin-B2

pP130

Protein Expression (Fold of Ctrl)

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p-Ephrin-B2

p-P130

α-Tubulin

p-SRC

p-FAK

α-Tubulin

E

Proliferation of HUVEC (% of control)

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Protein Expression (Fold of Ctrl)

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p-P130

p-SRC

p-FAK

α-Tubulin

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Clinical Cancer Research

Eph-B2/ephrin-B2 interaction plays a major role in the adhesion and proliferation of Waldenstrom’s Macroglobulinemia

Feda Azab, Abdel Kareem Azab, Patricia Maiso, et al.

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