Anatomy and Pathology/Oncology

Targeting the Platelet-Derived Growth Factor-beta Stimulatory Circuitry to Control Retinoblastoma Seeds

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PURPOSE. Vitreous seeding remains the primary reason for treatment failure in eyes with retinoblastoma (Rb). Systemic and intra-arterial chemotherapy, each with its own inherent set of complications, have improved salvage rates for eyes with advanced disease, but the location and biology of vitreous seeds present a fundamental challenge in developing treatments with minimal toxicity and risk. The aim of this study was to target the platelet-derived growth factor (PDGF)-PDGF-receptor β (PDGFRβ) signaling pathway and investigate its role in the growth of Rb seeds, apoptotic activity, and invasive potential.

METHODS. We performed ex vivo analyses on vitreous samples from Rb patients that underwentenuclcation and from patient-derived xenografts. These samples were evaluated by quantitative PCR, immunohistochemistry, and ELISA. The effects of disruption of the PDGF-PDGFRβ signaling pathway, both by pharmacologic and genomic knockdown approaches, were evaluated in vitro by cell proliferation and apoptotic assays, quantitative PCR analyses, Western blotting, flow cytometry, and imaging flow cytometry. A three-dimensional cell culture system was generated for in-depth study of Rb seeds.

RESULTS. Our results demonstrated that PDGFRβ signaling is active in the vitreous of Rb patients and patient-derived xenografts, sustaining growth and survival in an AKT-, MDM2-, and NF-kB-dependent manner. The novel three-dimensional cell culture system mimics Rb seeds, as the in vitro generated spheroids have similar morphologic features to Rb seeds and mimicked their natural physiology.

CONCLUSIONS. Targeting the PDGFRβ pathway in vitro reduces Rb cell growth, survival, and invasiveness and could augment current therapies. This represents a novel signaling pathway for potential targeted therapy to further improve ocular survival in advanced Rb.

Keywords: retinoblastoma, PDGFR, vitreous seeds, imatinib, molecular medicine

The greatest challenge in treatment of retinoblastoma (Rb) is the recurrence or resistance of vitreous seeding to current therapies. Overall, patients that have vitreous seeding have a poorer prognosis for ocular salvage.1–6 Although the mechanism controlling refractory or recurrent vitreous disease is unclear, it is likely related to a unique property of Rb cells that allows them to adapt and propagate in semisolid or liquid microenvironments, such as the vitreous and subretinal compartments.7

Understanding the tumor microenvironment is an important step in achieving overall treatment goals for Rb, including patient survival, globe salvage, and vision preservation.6,8 The vitreous body is a translucent medium in the eye composed of liquid (99%) and solid (1%) phases. Recent research has identified the presence of some inflammatory, mitogenic, and immunosuppressive mediators in the vitreous9–11 capable of promoting homeostasis as well as pathologic processes, most notably diabetic retinopathy and proliferative vitreous retinopathy resulting from complex rhegmatogenous retinal detachments.10,12–14 Platelet-derived growth factor (PDGF) has been identified as one such key regulator.13,15 Based on this evidence, we hypothesize that cellular infiltrates secrete PDGF
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in the vitreous, which may provide mitogenic activity to Rb cells via the PDGF-receptor (PDGFR).16–20 We found that reducing PDGFβ signaling in Rb tumor cells in vitro reduces growth, cell survival, and invasion potential via the AKT, MDM2, and NF-κB signaling pathways.

METHODS

Ethics Approval and Consent to Participate

The institutional review board at SJCRH and The University of Tennessee Health Science Center (UTHSC) approved all experiments involving human subjects. Informed consent was obtained cases where vitreous was harvested at the time of enucleation, an optional research objective that is part of an on-going prospective clinical trial (ClinicalTrials.gov, NCT01783535). This is in full compliance with and adheres to the tenets of the Declaration of Helsinki. The Institutional Animal Care and Use Committee at SJCRH approved all animal experiments. All protocols followed the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, in addition to the guidelines for laboratory animal experiments.

Cell Lines

Y79 (ATCC HTB-18) and Weri-Rb1 (ATCC HTB-169) cell line information and culture media were previously described.23

Vitreous Samples

Eyes were trephined after enucleation without disruption of underlying structures, which is important for diagnostic and staging purposes. Vitreous was aspirated using an 18-gauge needle and a 3-mL syringe. Vitreous from healthy controls were purchased from BioreclamationIVT (Baltimore, MD, USA). Lyophilized recombinant human PDGF-BB (10 μg) was purchased from Thermo Scientific. Vitreous from Rb patients treated at St. Jude Children’s Research Hospital (SJCRH) were fixed, paraffin-embedded, and serially sectioned. The probes anti-human PDGFRβ (16868; Abcam, Cambridge, MA, USA) and anti-human p-PDGFRβ (sc-339; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) were used. Patient-derived orthotopic xenografts (PDX) for Rb were injected into the eyes of SCID (NOD.CB17-Prkdcscid/J) mice. They were 339; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) were used.

Reagents

Gleevec was purchased in its generic chemical form, imatinib mesylate (IM; PubChem ID, 123596), from Sigma-Aldrich (Saint Louis, MO, USA). Lyophilized reagent was dissolved in deionized, distilled water to 10 mM and stored at –20°C. Lyophilized recombinant human PDGF-BB (10 μg) was purchased from Thermo Scientific (Waltham, MA, USA), dissolved in 1 mL 100 mM acetic acid with 0.1% BSA, and stored at –20°C.

Immunohistochemistry

Enucleated eyes from Rb patients treated at St. Jude Children’s Research Hospital (SJCRH) were fixed, paraffin-embedded, andserially sectioned. The probes anti-human PDGFRβ (16868; Abcam, Cambridge, MA, USA) and anti-human p-PDGFRβ (sc-339; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) were used. Patient-derived orthotopic xenografts (PDX) for Rb were described before.24 Briefly, tumors were harvested from the eyes of patients primarily enucleated for Rb. The samples were taken from a diverse patient population, with respect to clinical presentation, sex, and race. Samples were dissociated with trypsin, collected, and resuspended in a solution of RPMI and 10% fetal calf serum at 10,000 cells/μL. Approximately 50,000 cells were injected into the eyes of SCID (NOD.CB17-Prkdcscid/J) mice.

MTS Cell Proliferation Assay

Cell proliferation was investigated using the CellTiter96 AQueous One Solution Cell Proliferation Assay reagent (Promega, Madison, WI, USA), as described before25 by plating Y79 cells at a density of 5.0 × 10³ cells per well.

Protein Assays

PDGF-AB/PDGF-BB/VEGF ELISA. Vitreous samples were diluted 1:10 with assay buffer from the respective Invitrogen Human PDGF ELISA Kit (Thermo Scientific). Diluted samples were placed in an antibody-coated microwell plate along with appropriate standards following manufacturer’s protocols. Absorbance calculated using both 450 nm and 550 nm as reading wavelengths. Assays were conducted in triplicates.

Cleaved Caspase-3 ELISA. A caspase-3 (active) ELISA kit (Thermo Scientific) was used according to manufacturer’s instructions: 30 μg of total extracted protein was incubated in a microplate well in triplicate at room temperature (RT) for 2 hours. Absorbance values were calculated as above.

Western Blot Analyses. We followed our published protocols25,26 for electrophoresis. Membranes were blocked with 5% BSA in Pierce Tris Buffered Saline Tween-20 for 1 hour, followed by incubation with primary antibodies overnight at 4°C. HRP-linked secondary antibodies were added and incubated at RT for 1 hour. Signal was detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). The endogenous control was either β-ACTIN or GAPDH (both from Cell Signaling Technology [CST], Beverly, MA, USA). Experiments were conducted in triplicate. The primary antibodies were anti-PDGFRβ (sc-432; 1:100), anti-phosphorylated PDGFRβ (sc-373805; 1:100), anti-VEGF (sc-53462; 1:200), anti-FK-1 (also known as [aka] VEGFR2, sc-6251; 1:200), anti-p-FK-1 (aka p-VEGFR2, sc-101821; 1:100), anti-MDM2 (sc-965; 1:200), anti-phosphorylated MDM2 (sc-53568; 1:200), anti-AKT (catalog no. 9272; CST; 1:1000), anti-phosphorylated AKT (catalog no. 4058; CST; 1:1000), anti-BCL-2 (catalog no. 2870; CST; 1:1000), anti-GAPDH (catalog no. 8884; CST; 1:1000). Secondary antibodies were anti-mouse IgG HRP-linked (catalog no. 7076; CST; 1:1000) and anti-rabbit IgG HRP-linked antibodies (catalog no. 7074; CST; 1:1000).

Flow Cytometry Studies

Apoptosis. Annexin-V and propidium iodide (PI) were used as before.27 Data were acquired using a BD LSR II Cytometer (BD Biosciences, San Jose, CA, USA) with BD FACSDiva software; analysis was performed using FlowJo v.X.0.0.8 (FlowJo, LLC, Tree Star, Ashland, OR, USA). Samples were analyzed in triplicate.

PDGFβR Expression. Y79 Rb cells were labeled with anti-PDGFRβ (sc-432; 1:50) on ice for 30 minutes, followed by incubation with a donkey anti-rabbit Alexa Fluor 647 (Thermo Scientific; 1:50) secondary antibody for 20 minutes. Cells were fixed with 2% paraformaldehyde for 20 minutes prior to analysis. Analyses of PDGFβR from three-dimensional (3D) assays were conducted as follows: spheroids were transferred to a fluorescence-activated cell sorting (FACS) tube by using a magnetic pen, followed by spheroid disruption by pipetting. Cells were labeled as above. Controls included an isotype control and unlabeled samples. Single label controls were set up using The AbC Total Antibody Compensation Bead Kit (Thermo Scientific). Data acquisition was done in a ZE5 Cell Analyzer from Propel Labs (Fort Collins, CO, USA). Analysis was done as above.

p65 NF-κB Nuclear Translocation by Imaging Flow Cytometry. Collected cells were fixed in 2% paraformaldehyde and permeabilized in 0.01% Triton X-100. Samples were blocked with PBS/1% fetal bovine serum before addition of anti-p65 (catalog no. 8242; CST; 1:100) antibody for 1 hour on ice. Cells were washed with PBS, and donkey anti-rabbit Alexa...
Fluor 488 (Thermo Scientific; 1:100) secondary antibody was added for 1 hour. Nuclei were labeled with DRAQ5 (BioLegend, San Diego, CA, USA; 1:100). Cells were analyzed using the Amnis FlowSight Imaging Cytometer (EMD Millipore, Seattle, WA, USA). Data were analyzed using IDEAS software (EMD Millipore). Mean fluorescent intensity (MFI) was measured to investigate levels of p65 as well as the percentage of cells with p65 translocation to the nucleus. The fluorescence controls were single labeled cells acquired with both 488 nm and 642 nm. We collected a minimum of 10,000 events (cells) from each sample, and experiments were done in triplicate.

**Cell Invasion Assay.** A CytoSelect 24-well cell invasion kit (Cell BioLabs, Inc., San Diego, CA, USA) was used to measure invasion of Y79 cells as described previously. The synthesized cDNA was preamplified with TaqMan PreAmp Master Mix. Gene expression assays, nuclease-free water, and TaqMan Universal Master Mix were loaded into each well. Plates were processed via Roche LightCycler 480, and the results were analyzed according to the comparative ΔΔT method as described before.

**siRNA Transfections.** Y79 cells were plated overnight in 6-well plates at a final density of 3.0 × 10⁵ cells per well in 2 mL RB media (without antibiotics), following manufacturer’s guidelines. Lypophilized PDGFRB siRNA duplex (sc-29942) was diluted in nuclease-free water to a final concentration of 10 μM, following manufacturer’s instructions. A total of 0.6 μg of PDGFRB siRNA was diluted in 100 μL of siRNA transfection medium (Santa Cruz Biotechnology, Inc.) per well (solution A). In parallel, 6 μL of siRNA transfection reagent was added into 100 μL siRNA transfection medium (solution B) per well. Solution A and solution B were combined and incubated at RT for 30 minutes. Meanwhile, Y79 cells were harvested, washed in transfection medium and resuspended in 800 μL of siRNA transfection medium per well, following the addition of solution A and B. Cells were incubated for 6 hours at 37°C/5% CO2. At this point, 1 mL of RPMI/20% fetal bovine serum was added, and cells were incubated for 18 hours prior to performing functional assays. As a control, we used a scramble sequence that is known to not target any specific oligonucleotides.

**RESULTS**

**Expression of the PDGFR Signaling Network in Rb Tumors and Cell Lines**

We investigated the nonphosphorylated and the phosphorylated (p-PDGFRβ) expression of PDGFRβ in primary human Rb samples from enucleated eyes of Rb patients with advanced intraocular disease with vitreous seeds. Our representative results from a cohort of 15 different samples (Figs. 1A–D) demonstrated an abundant p-PDGFRβ labeling, compared with the nonphosphorylated form. We measured similar mRNA expression compared with the nonphosphorylated PDGFRβ in vitreous seeds. The human orthotopic xenograft for Rb has been already established as a comparable model to Rb disease. Using this system, we investigated the expression of PDGFRβ in samples from PDX. As shown in Figures 1G and 1H, there is no. 11

**qPCR Analysis.** A final 10-μL mixture of the preamplified cDNA, gene expression assays, nuclease-free water, and TaqMan Universal Master Mix were loaded into each well. Plates were processed via Roche LightCycler 480, and the results were analyzed according to the comparative ΔΔT method as described before. In parallel, Y79 measured a higher expression of PDGFR compared with the PDGFR mRNA (Fig. 1J, left). In contrast, Weri-1 Rb cells measured similar PDGFR and PDGFR mRNA expression.
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Results are represented as mean ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001.

**FIGURE 1.** Expression of the active and nonactive forms of PDGFRβ in Rb. (A–D) Representative images of immunohistochemical staining for expression of nonphosphorylated PDGFRβ and p-PDGFRβ (active) from a cohort of 15 enucleated eyes of Rb patients. Images taken at 20×. (E, F) Representative image of immunohistochemical results of PDGFRβ in vitreous seeds. Images taken at 40×. (G, H) Images from PDX. Images taken at 20×. (I) qPCR analysis of mRNA from PDX for key members of PDGF signaling pathway. Results are represented as mean ± SEM fold change of the target gene over HPRT1, the housekeeping gene. (J) mRNA expression of both PDGFRα and PDGFRβ in both Rb cell lines Y79 and Weri-Rb. Results are represented as mean ± SEM fold change of the target gene over HPRT1, the housekeeping gene. (K, L) Levels of PDGF-AB (K) and PDGF-BB (L) in vitreous samples from healthy controls vitreous compared to those of Rb patients by ELISA analyses. n = 9 in PDX mRNA analyses with 4 replicates per sample; n = 4 for Y79 and Weri-1 mRNA analyses with 4 replicates per sample; n = 6 in ELISA analyses done in triplicates. All results represent mean ± SEM; **P = 0.0010, ***P = 0.00051.

(Fig. 1J, right). Because vitreous seeds are considered an aggressive phenotype, we focused the rest of the in vitro studies on Y79 Rb cells.

To investigate the presence and abundance of PDGF proteins that signal through PDGFRβ in the vitreous microenvironment, we collected vitreous samples from eyes of Rb patients that underwent primary enucleation and compared the protein levels with those of healthy controls. We discovered PDGF-AB (Fig. 1K) to be more abundant in the Rb samples compared with those of healthy controls (**P = 0.0010). Similarly, PDGF-BB levels were higher in the Rb samples compared with healthy controls (**P = 0.00051). The levels of PDGF-BB in the Rb samples were >5-fold higher than the healthy controls (Fig. 1L). Our work reveals higher levels of active PDGFRβ as well as high levels of the receptor’s ligands in Rb samples compared with healthy controls.

**Pharmacologic Disruption of PDGFRβ Signaling Reduced Rb Proliferation, Invasion, and Increased Cell Death**

We used IM, which selectively inhibits the tyrosine kinase activity of the PDGFRβ protein tyrosine kinase,31,32 to disrupt the PDGF-PDGFRβ signaling pathway and investigate the role(s) it may play in Rb. We previously determined the most efficacious dose at inhibiting cellular proliferation was 10 μM (data not shown). The disruption of the PDGFRβ signaling pathway was confirmed by Western blotting (Fig. 2A). To mimic the physiology of the disease in the vitreous microenvironment, we added recombinant human PDGF (rhPDGF) to cell cultures. We measured increased levels of PDGFRβ activity when rhPDGF was added (**P = 0.0073). In contrast, there was a striking reduction of PDGFRβ activity when cells were treated with IM (**P = 0.0046) or rhPDGF + IM (**P = 0.0015) compared with rhPDGF. Next, we quantified the percentage of Y79 PDGFRβ+ cells by flow cytometry analysis and found a significant reduction of Y79 PDGFRβ+ after 48 hours of treatment with IM compared with untreated (**P = 0.0019) and rhPDGF-treated cells (**P = 0.0024), as shown in Figure 2B, left. Representative histograms showing the percentage of PDGFRβ+ Y79 cells are shown in Figure 2B, right. We measured cell viability by the MTS cellular proliferation assay. The results showed a significant increase in proliferation over time in the presence of rhPDGF, illustrating a potential mitogenic role. This mitogenic effect was inhibited by IM (Fig. 2C; **P = 0.02, ***P = 0.003, **P = 0.0016, ***P < 0.0005). We then investigated if inhibition of PDGFRβ could increase cell death (Fig. 2D) by flow cytometry analysis of apoptotic (annexin V+PI-) cells. At the time of setting up the cell cultures, we measured cell death by colabeling of annexin V and PI and found about 3% cell death (Fig. 2D, right). There was an increase in cell death in IM and rhPDGF + IM compared with untreated and rhPDGF (**P = 0.0021, **P = 0.0033). These results illustrated the role of the PDGFRβ signaling in Rb cell growth and death.

We measured the invasive potential of Y79 cells by culturing them in well inserts coated with basement membrane through use of a colorimetric assay (Fig. 2E) and by quantitation of cells per field (Fig. 2F). There was a reduction in the colorimetric measurements of IM and rhPDGF + IM-treated cells that crossed the membrane (**P = 0.0304, **P = 0.0070) compared to those untreated and treated with rhPDGF. This data was confirmed with the quantitative analysis. IM-treated (**P = 0.0357, **P = 0.0061) and rhPDGF + IM-treated (**P = 0.0118) cells showed less invasion compared with untreated and rhPDGF cells. Representative images of cells in each condition are shown in Figure 2G.
Disruption of PDGFRβ Targets the AKT and NF-κB Signaling Cascades

AKT and NF-κB signaling, which are linked to antiapoptotic mechanisms, have not been studied within the PDGFRβ pathway in Rb. Qualitative PCR analysis in Figure 3A demonstrated downregulation of MDM2 mRNA after disruption of the PDGFRβ pathway compared with rhPDGF-treated cells relative to untreated cell cultures by using HPRT1 as housekeeping gene. A similar effect was measured when MDM4 mRNA (Fig. 3B) was tested. As shown in Figure 3C, MDM2 signaling is impaired in IM-treated (P = 0.0075) and rhPDGF + IM-treated (P = 0.0015) cells compared with untreated and rhPDGF. We investigated if the reduction in cell proliferation is AKT-dependent. A small, albeit significant (P = 0.0054), difference was found in AKT signaling in rhPDGF + IM-treated cells (Fig. 3D).

After examination of MDM2 and AKT, both involved in cell survival pathways, we investigated the mechanisms by which PDGFRβ controls cell death in Rb. Figure 3E reveals Y79 cells treated with IM and rhPDGF + IM have a significant reduction in BCL-2 levels compared with untreated and rhPDGF by Western blot analyses (***P = 0.001; **P = 0.0002). We sought to test the levels of cleaved caspase-3 by ELISA to investigate if the BCL-2 reduction was concomitant to an increase in cleaved caspase-3 (P = 0.007) compared with the rest of the cell culture conditions.

Our next investigation aimed to evaluate if PDGFRβ signaling could exert an inhibitory effect in VEGF-VEGFR2 signaling, which is expressed in Rb.26,33,34 We confirmed the expression of VEGFA mRNA in Y79 cells and in the PDX model of Rb (Figs. 3G, 3H). Additional qPCR analyses revealed no significant difference in VEGFA and VEGFR2 mRNA expression after disruption of the PDGFRβ signaling (Figs. 3I, 3J).
FIGURE 3. PDGFRβ signaling regulates key downstream signaling mediators. qPCR analyses on mRNA levels of (A) MDM2 and (B) MDM4 relative to untreated Y79 cells under the cell culture conditions described above. HPRT1 used as housekeeping gene. Western blot analyses (C) of MDM2 activity and (D) AKT activity after disruption of the PDGFRβ signaling cascade. IM, *P = 0.0275; rhPDGF + IM, **P = 0.0011; AKT, *P = 0.0054. Evaluation of the antiapoptotic mediator (E) BCL-2 by Western blot (**P = 0.001 and ***P = 0.0002); and the proapoptotic cleaved (active) caspase-3 by ELISA (F), *P = 0.007. (G–L) Downregulation of the VEGFR signaling when PDGFRβ is inhibited. (G) VEGFA mRNA levels were measured by qPCR analysis in both Y79 cells and (H) PDX samples. (I) Both VEGFA and (J) VEGFR2 mRNA levels were measured across respective treatments as well as (K) Western blot analyses of VEGFR2 activity; *P = 0.0191. (L) Measurement of VEGFA levels in a cohort of vitreous samples from healthy controls compared to vitreous of Rb patients. ****P < 0.0001. (M–O) Assessment of the NF-κB signaling. Representing images of Y79 cells (M) labeled and analyzed for nuclear (labeled with DRAQ5) translocation of the p65 subunit of NF-κB (AlexaFluor 488 conjugated); ***P < 0.001 (N) the percentage of treated Y79 cells with nuclear localization of the p65 subunit and *P = 0.0271 (O) the expression (or MFI) of the p65 subunit using an Amnis FlowSight Imaging Cytometer. Experiments from A–G and I–K were done with n = 3 and each sample done in triplicates. Experiment from H, n = 9 and each sample done in replicates of 4. n = 6 in ELISA analyses done in triplicates. Experiments from M–O tested 10,000 cells per condition. All results represent mean ± SEM.
no changes at the transcriptional level, there was a reduction in VEGFR2 signaling after treatment with IM (*P = 0.0191) compared with the controls, as shown in Figure 3K. These results suggest IM treatment could impact the abundance of VEGF protein in the vitreous microenvironment, which is shown in Figure 3L to be higher in Rb samples compared with healthy controls (***P = 0.0001). Taken together, these results demonstrated that targeting of PDGFRβ signaling impairs VEGFR2 signaling.

The disruption of PDGFRβ signaling via IM showed this pathway plays a role in Rb tumor cell survival via AKT. We hypothesized whether the suppression of Y79 survival may occur via a crosstalk between AKT and NF-κB. To address this, we used imaging flow cytometry to measure the nuclear translocation of the NF-κB p65 subunit (Figs. 3M–O). Quantitative analysis showed a distinct reduction in the percentage of cells showing p65 nuclear translocation (Fig. 3M) after IM treatment (**P < 0.001) compared with the untreated and rhPDGF controls. Along with a reduction on the percentage of cells showing p65 nuclear translocation, we measured an overall reduction in the p65 protein by measurement of the MFI (Fig. 3N) in the presence of IM (P = 0.0271) compared with the controls. Representative images of the p65 nuclear localization analyses are shown in Figure 3O. Colocalization of p65 (green) to the nucleus (red) is depicted in yellow. Collectively, our work supports our hypothesis that the PDGFRβ plays an essential role in Rb cell survival and death via AKT and NF-κB.

Reduction of Rb Spheroids by IM in a 3D Cell Culture Model of Rb Vitreous Seeds

There is a lack of in vitro models for Rb vitreous seeds. We adapted our in vitro 3D cell culture system to recreate Rb seeds in a vitreous-like microenvironment for an in-depth study. Schematics of the procedure are shown in Figures 4A–F. Rb cells are mixed with nanoshuttles (Fig. 4B) between 5 to 12 hours at 37°C/5% CO₂. These nanoshuttles are composed of poly-L-lysine, iron, and gold. Multiple aggregates or clusters of cells are found within the cell cultures. Cells are magnetized using a magnet, as depicted in Figure 4C. As “seeds” are generated by magnetic levitation, the aggregates are disrupted and plated. An additional magnet, which varies by type of assay, is applied for 30 minutes (Fig. 4D). Using the 3D system, we are able to generate a spheroid that forms one large mass visible to the naked eye (Figs. 4E, 3F). Quantitative analysis of live cells by trypan blue exclusion, shown in Figure 4G, revealed no difference (P = 0.430) in the viability of cells after overnight culture. We compared light microscopy images of the Rb spheroids to Rb seeds and found similar morphology (Fig. 4H). We then measured the area of the spheroids over time after treatment with IM using ImageJ software and
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A recent study by Sanft and colleagues demonstrated the presence of the PDGFRβ in Rb samples by immunohistochemistry. Our study demonstrates increased activity of the PDGFRβ signaling network ex vivo in primary human Rb samples from...
enucleated eyes with advanced intraocular disease with vitreous seeds. We demonstrated this stimulatory circuitry by the high expression of PDGFB mRNA in Rb tumor cells, the levels of active PDGFRβ signaling, and the abundance of the PDGFRβ ligands in the vitreous microenvironment of Rb patients.

We recapitulated these findings in vitro by using the Y79 Rb cell line, which is considered to be the aggressive model of the disease. The in vitro system allowed us to demonstrate the role of the PDGF-PDGFRβ signaling pathway in Rb by using the selective tyrosine kinase inhibitor IM. Our mechanistic studies measured reduction of cell viability and invasive potential after disruption of the PDGF-PDGFRβ by IM in Y79 cells. These events were associated with a reduction of antiapoptotic mechanisms in the cell, including downregulation of MDM2 mRNA, as well as reduced signaling activity of MDM2, AKT, the p65 subunit of NF-kB, and BCL-2. In addition, VEGF2 signaling after disruption of PDGFRβ signaling with IM was noted. We postulate PDGF-PDGFRβ signaling may help regulate VEGF production in the tumor microenvironment, which is abundant under pathologic conditions.

Current investigations using IM in cancer, specifically in glioblastoma multiforme, suggest a clinically safe application in the pediatric population and penetration across the blood-brain barrier (BBB). The blood-retinal barrier is the ocular counterpart of the BBB. Emerging evidence shows IM is also capable of reducing neuroinflammation in autoimmune disorders and restores the integrity of the BBB in a rat model of MS.

Our limited understanding of vitreous seeds and the vitreous microenvironment has precluded the advancement in the development of novel therapies to specifically address this aspect of Rb. We generated a novel 3D Rb cell culture system based on the magnetic levitation studies originally from Haisler and colleagues to develop tumor seeds in vitro. When these spheroids were examined morphologically, they were similar to in vivo Rb vitreous seeds. Munier and colleagues recently outlined the three main subtypes of vitreous seeding patterns; each subtype may indicate different biologic properties of disease and potential different responses to conventional therapy. Amram et al recently described the histopathology associated with each subtype and concluded spheres to be the most aggressive subtype. We compared our in vitro spheroids to the morphologic characteristics described in Amram’s studies and discovered similarities to the spheres subtype, specifically the translucent center with some spheroids exhibiting Rb cell detachment from the outer area. This validation of our in vitro 3D system supported further study of Rb spheroids.

In this investigation, we demonstrated preclinically that PDGF from both autocrine and paracrine sources signals through the PDGFRβ to sustain Rb growth in an avascular system, such as the vitreous. Targeting the PDGFRβ could increase the sensitivity of these tumor cells to current treatments, as current therapies have failed to address the reduced proliferative capacity and metabolism of these unique tumor seeds.

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