SHORT COMMUNICATION

Glioma-Derived Platelet-Derived Growth Factor-BB Recruits Oligodendrocyte Progenitor Cells via Platelet-Derived Growth Factor Receptor- α and Remodels Cancer Stroma

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Glioma is an aggressive and incurable disease, and is frequently accompanied by augmented platelet-derived growth factor (PDGF) signaling. Overexpression of PDGF-B ligand characterizes a specific subclass of glioblastoma multiforme, but the significance of the ligand remains to be elucidated. For this end, we implanted a glioma-cell line transfected with PDGF-BB—overexpressing vector (GL261-PDGF-BB) or control vector (GL261-vector) into wild-type mouse brain, and examined the effect of glioma-derived PDGF on the tumor microenvironment. The volume of GL261-PDGF-BB rapidly increased compared with GL261-vector. Recruitment of many PDGF receptor (PDGFR)- α and Olig2-positive oligodendrocyte precursor cells and frequent hemorrhages were observed in GL261-PDGF-BB but not in GL261-vector. We then implanted GL261-PDGF-BB into the mouse brain with and without *Pdgfra* gene inactivation, corresponding to PDGFR α -knockout (KO) and Flox mice, respectively. The recruitment of oligodendrocyte precursor cells was largely suppressed in PDGFR α -KO than in Flox, whereas the volume of GL261-PDGF-BB was comparable between the two genotypes. Frequent hemorrhage and increased IgG-leakage were associated with aberrant vascular structures within the area where many recruited oligodendrocyte precursor cells accumulated in Flox. In contrast, these vascular phenotypes were largely normalized in PDGFR α -KO. Increased matrix metalloproteinase-9 in recruited oligodendrocyte precursor cells and decreased claudin-5 in vasculature may underlie the vascular abnormality. Glioma-derived PDGF-B signal induces cancer stroma characteristically seen in high-grade glioma, and should be therapeutically targeted to improve cancer microenvironment. (*Am J Pathol* 2016, 186: 1081–1091; <http://dx.doi.org/10.1016/j.ajpath.2015.12.020>)

Glioblastoma multiforme (GBM), World Health Organization grade IV astrocytoma, is the most common malignant brain tumor of adults. GBM is characterized by highly invasive features and prominent vascular involvement.¹ In addition to the aggressive growth of tumor cells, the associating secondary events, like intracranial hemorrhage, are critical prognostic factors as well.² Recently, tumor cells per se as well as microenvironment—defined by extracellular matrix and tumor stromal cells, including vascular endothelial cells and glial cells—gained attention as a therapeutic target.^{3,4} Typically, a

treatment targeting tumor angiogenesis using an antibody against vascular endothelial growth factor, bevacizumab, is used at tumor recurrence, and achieves significant improvement of the disease.⁵ Furthermore, the current efforts are developing novel and potent agents that target multiple angiogenic pathways, including platelet-derived growth factor (PDGF) and

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fibroblast growth factor, for the treatment of advanced solid tumors.⁶

PDGF family members include PDGF-A, PDGF-B, PDGF-C, and PDGF-D, which are assembled as disulfide-linked homodimers or heterodimers. The PDGF signal is mediated by two types of PDGF receptors, PDGF receptor α (PDGFR α) and PDGFR β .⁷ PDGF ligands and receptors were expressed in the brain; among them, PDGFR α is most abundantly expressed in oligodendrocyte progenitor cells (OPCs), and is implicated in the regulation of the proliferation, migration, and differentiation of OPCs in developing brain.⁸ Furthermore, in the recent studies, PDGFR α signaling is well documented in the disruption of the blood-brain barrier in different pathological conditions.^{9,10}

PDGF autocrine mechanisms are assumed to be most important in glioma,¹¹ and proteomic analyses demonstrated that the overexpression of PDGF-B with increased PDGFR β phosphorylation characterized a specific subclass of GBM that comprises nearly 30% of human GBM.¹² Because PDGF-B activates PDGFR- α and PDGFR- β and is a chemoattractant of glial and mesenchymal cells,¹³ PDGF-B overexpression in GBM may chemoattract a range of intermingling nonneoplastic parenchymal cells that were characteristically seen in high-grade brain tumors.¹⁴ Along this line, many PDGFR α -positive OPC-like cells were recruited toward PDGF-B-induced rodent glioma.¹⁵ However, the mechanism of GBM-derived PDGF-B to induce cancer stroma, and the functional implication of tumor microenvironment defined by cancer stroma, remains to be elucidated for the establishment of a novel therapeutic target of GBM.

In the present study, we hypothesized that GBM-derived PDGF-B induces tumor microenvironment and may contribute to the aggressive behavior of GBM. For this end, we developed a PDGF-BB-overexpressing glioma cell line (GL261-PDGF-BB) and implanted them into the mouse brain. Furthermore, the same transplantation was conducted after the inactivation of host PDGFR α signaling by conditionally induced *Pdgfra* gene inactivation. Paracrine PDGF signal from glioma cells was shown to recruit many OPCs, and the recruited OPCs were implicated in the regulation of vascular leakage and hyperplasia with remodeling of extracellular matrix.

Materials and Methods

All experimental animal procedures were approved by the Committee for Institutional Animal Care and Use at the University of Toyama (Toyama, Japan). All of our study protocols were approved by the Ethics Committee of the University of Toyama.

The Generation of *Pdgfra* Conditionally Inactive Mice

Mutant mouse harboring a genetically mutated *Pdgfra* gene, in which exons 4 and 5 of *Pdgfra* were flanked by

two loxP sequences (*Pdgfra*^{lox/lox}), was generated as follows. Briefly, a bacterial artificial chromosome genomic clone (RP24-148N4), originating from the DNA of a C57BL/6 mouse and containing *Pdgfra*, was obtained from the BACPAC Resource Center CHORI. The constructed targeting vector included a DNA fragment containing a loxP sequence and *pgk*-Neo cassette flanked by two FLP recognition target (*frr*) sites¹⁶ and pMC1DTABGHA with a bovine growth hormone-derived polyadenylation signal sequence.¹⁷ The embryonic stem cell line RENKA, derived from the C57BL/6N strain,¹⁸ was used and the resulting targeting vector was electroporated, as described previously.¹⁹ The obtained male chimeric mouse was crossed with a female CAG-FLPe deleter mouse²⁰ to remove the *pgk*-Neo selection cassette and obtain the heterozygous *Pdgfra*^{lox/+} strain. Genotyping was performed by PCR using the following primers: forward, 5'-ATGCCAACTCTGCCTGATTGA-3' and reverse, 5'-CTCACGGAACCCCCACAAC-3'. Flox mice were crossbred with chicken β -actin-promoter/CMV-enhancer-driven *Cre*-transgenic mice (*CAGG-CreER*^{+/+}; The Jackson Laboratory, Bar Harbor, ME; CreER mouse)²¹ that harbor the fusion gene Cre recombinase and estrogen receptor (CreER). The resulting offspring were mice harboring *CAGG-CreER*^{+/+};*Pdgfra*^{lox/lox} (CreER-Flox mice) or *Pdgfra*^{lox/lox} (Flox mice). Mice with a systemic *Pdgfra* inactivation [PDGFR α -knockout (KO) mice] were obtained by tamoxifen (Sigma-Aldrich, St. Louis, MO) administration to CreER-Flox mice. Flox mice were treated identically and used as controls.

Generation of PDGF-BB Overexpression Glioma Cell Line

To monitor the cells expressing the mouse PDGF-BB, we introduced IRES-EGFP DNA fragment from pIRES2-EGFP (Clontech, Mountain View, CA) after the coding region of mouse *Pdgfb* expression plasmid pBLAST49-mPDGFB (full) v24 (InvivoGen, San Diego, CA). The 0.12-Kbp DNA fragment containing mouse *Pdgfb* was amplified with primers PIE-U1 (5'-GGGGAATTCGGTGACCATTTCGGACGGTGAG-3') and PIE-L1 (5'-GGGGATCCCTAGGCTCCGAGGGTCTCC-3') using pBLAST49-mPDGFB (full) v24 as a template. The amplified DNA fragment was subcloned into the EcoRV site of the pLITMUS28 (New England Biolabs, Herts, UK) to yield the plasmid pLITPIE. The 0.12-Kbp EcoRI-BglII DNA fragment from pLITPIE was cloned between EcoRI and BamHI sites of the pIRES2-EGFP to yield the plasmid pIRES-PIE. The 1.4-kb BstEII-NotI (blunt) fragment from pIRES-PIE was ligated with the 3.6-kb BstEII-NheI (blunt) DNA fragment from pBLAST49-mPDGFB (full) v24 to yield the plasmid pPIE. Plasmid pPIE and empty vector were transfected to the GL261 glioma cell line (kindly provided by Dr. Atsushi Natsume, Department of Neurosurgery, Graduate School of Medicine, Nagoya University, Nagoya, Japan) by electroporation (NEPA21 Electroporator; Nepa Gene, Chiba, Japan), according to the manufacturer's procedure. Transfected cells

were single cloned, and passaged several times to obtain stable expression clones. Resulting PDGF-BB overexpression and vector cell lines were termed GL261-PDGF-BB and GL261-vector, respectively.

Immunoblotting

Sample preparations and all other procedures for Western blot analysis have been described elsewhere.²² Briefly, cultured cells were lysed using radioimmunoprecipitation assay buffer (Thermo Fisher, Waltham, MA) on ice. Whole cell lysates were separated by SDS-PAGE and then electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were blocked for 1 hour at room temperature in a 5% milk containing 50 mmol/L Tris buffer with 150 mmol/L NaCl and 0.05% Tween 20. The membranes were then probed with rabbit anti-PDGFR α (1:1000; Santa Cruz Biotechnology, Dallas, TX), rabbit anti-PDGFR β (1:500; Merck Millipore, Billerica, MA), and mouse anti- β -actin (1:20,000; Sigma-Aldrich, St. Louis, MO) antibodies. The membranes were washed in a 50 mmol/L Tris buffer containing 150 mmol/L NaCl and 0.05% Tween 20, and were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies. Immunoreactive bands were detected using enhanced chemiluminescence reagents (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), according to the manufacturer's instructions.

RNA Extraction, cDNA Synthesis, and Real-Time PCR

Sample preparations and all other procedures for real-time PCR have been described elsewhere.²³ Briefly, total RNA was isolated from cultured cells using miRNA easy Mini Kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. Total RNA (150 μ g) was reverse transcribed, as detailed in the PrimeScript RT reagent kit protocol (Takara Bio Inc., Shiga, Japan). For real-time PCR performed with a Takara Thermal Cycler Dice Real Time System TP800 (Takara Bio Inc.), cDNAs were diluted 1:25 in the reaction mixture consisting of SYBR Premix EX Taq II (Takara Bio Inc.). The real-time PCR program consisted of hot start enzyme activation at 95°C for 10 seconds and 45 cycles of amplification at 95°C for 10 seconds and 60°C for 40 seconds. Finally, to obtain the dissociation curve, a final cycle was performed at 95°C for 1 minute, 60°C for 30 seconds, and then 95°C for 10 seconds. Primers were as follows: *Pdgfb*, forward, 5'-TGCTGAGCGACCACTC-CATC-3' and reverse, 5'-CTCGGGTCATGTTCAAGTCA-3'; *Pdgfra*, forward, 5'-AGCAAACATCTTGACTTG-GGAACA-3' and reverse, 5'-ACTTGCATCATTTCCCGGA-CAC-3'; *Pdgfrb*, forward, 5'-GAACGACCATGGCGAT-GAGA-3' and reverse, 5'-GCATCGGATAAGCCTCGA-ACA-3'; and *Actb*, forward, 5'-CATCCGTAAAGAC-CTCTATGCCAAC-3' and reverse, 5'-ATGGAGCCACC-GATCCACA-3'. For data analysis, mouse β -actin (*Actb*)

housekeeping gene was used as an internal control. Induction values were calculated using analysis software (Takara Bio Inc.).

Colony Formation Assay

GL261-PDGF-BB cells and GL261-vector cells were cultured in a soft agar-based three-dimensional culture system, Cyto-Select 96-Well *in Vitro* Tumor Sensitivity Assay (Cell Biolabs, San Diego, CA). Tumor cells were seeded at 5×10^3 cells per well in the 96-well plate, and were photographed under the conventional microscopy on day 1 after starting of culture. Six days later, tumor cell-derived colonies were microphotographed, and the growth rate was measured by OD 570 nm after MTT reagent reaction, according to the manufacturer's procedure.

Tumor Implantation and Tamoxifen Administration

Tamoxifen (Sigma-Aldrich) was orally administered once a day (225 mg/kg body weight) for 5 consecutive days, starting on 2 days before tumor implantation. Eight-month-old male mice were used for the experiments. All surgical procedures were conducted under anesthesia with sodium pentobarbital (i.p. injection, 100 mg·kg⁻¹). Using a small dental drill, a hole was made in the skull at the correct stereotaxic coordinates. After this, the dura matter was removed using small curved forceps, and exposure of the meninges was confirmed by observation of a small amount of cerebrospinal fluid at the exposed position. A needle (internal diameter, 0.13 mm) was set in the stereotaxic device (David Kopf Instruments, Tujunga, CA), connected to a Hamilton microsyringe via polyethylene tubes filled with phosphate-buffered saline, and set on a microsyringe pump (CMA 400; Harvard Apparatus, Holliston, MA). The needle was inserted to the targeted location in the striatum at 1.0, 2.0, and 3.0 mm at anteroposterior relative to bregma, mediolateral, and dorsoventral from surface of the brain, respectively. Tumor cell suspension (1 μ L; 1.0×10^5 cells· μ L⁻¹) was infused at a flow rate of 0.1 μ L·minute⁻¹. After the infusion, the needle was retained at the same position for 10 minutes to ensure no backflow. Under deep anesthesia with sodium pentobarbital (i.p. injection, 50 mg/kg body weight; Dainippon Sumitomo, Osaka, Japan), mice were transcatheterially perfused with phosphate-buffered saline, followed by perfusion and immersion in 4% paraformaldehyde at day 20 after tumor implantation.

Immunohistochemistry and Immunofluorescence Staining of Paraffin-Embedded Sections

For paraffin section preparation, brains were immediately fixed with 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4), dehydrated with a graded series of ethanol solutions, and embedded in paraffin. The sections (5 μ m thick) were subjected to either hematoxylin and eosin staining or immunostaining.

Figure 1 *In vitro* characterization of the platelet-derived growth factor (PDGF)-BB overexpression glioma cell lines. **A–C:** Real-time PCR analyses for the *Pdgfb* and *Pdgfrs* in glioma cell lines transfected with PDGF-BB–overexpressing vector (GL261-PDGF-BB) and with control vector (GL261-vector). Four clones were examined from GL261-PDGF-BB and GL261-vector, respectively. **A:** *Pdgfb* mRNA expression is much higher in GL261-PDGF-BB clones than in GL261-vector clones. **B:** *Pdgfra* mRNA expression is lower in GL261-PDGF-BB than in GL261-vector. **C:** *Pdgfrb* mRNA expression is comparable in all clones, except for one GL261-vector clone. **D:** Western blot analysis shows that PDGFR α is abundant in GL261-vector than in GL261-PDGF-BB. PDGFR β was undetected in all examined cell lines. The lysates from cultured mouse skin fibroblasts were used as positive controls. Equal sample loading was confirmed by β -actin. **E:** Representative photographs of GL261-vector and GL261-PDGF-BB cell lines in colony formation assay. **F:** Colony formation assay of four clones of GL261-PDGF-BB and GL261-vector. $n = 6$ from each clone (**A–C**). Scale bar = 200 μ m (**E**). AU, arbitrary unit.

Immunohistochemistry and immunofluorescence were performed, as described previously.^{24,25} Briefly, the sections were incubated at 4°C overnight with the following primary antibodies: goat polyclonal anti-PDGFR α (1:1000; Neuromics, Edina, MN), goat polyclonal anti-PDGFR β antibody (1:100; R&D, Minneapolis, MN), rabbit polyclonal anti-Olig2 (1:500; IBL, Hamburg, Germany), goat polyclonal anti-Sox10 (1:100; Santa Cruz Biotechnology), rat monoclonal anti-CD31 (1:100; Dianova, Hamburg, Germany), rabbit polyclonal anti-collagen type IV (1:500; Millipore, Darmstadt, Germany), rabbit polyclonal anti–matrix metalloproteinase (MMP)-9 (1:100; Abcam, Cambridge, UK), and rabbit polyclonal anti–claudin-5 (1:100; Invitrogen, Carlsbad, CA). Colorimetric immunostaining was conducted using the appropriate Histofine Simple Stain Mice System (Nichirei Biosciences, Tokyo, Japan) and 3,3'-diaminobenzidine tetrahydrochloride (Dako, Glostrup, Denmark) reaction. The leakage of serum protein was detected by immunohistochemistry for IgG using an anti-IgG detection system from Histofine Simple Stain Mice System (Nichirei Biosciences), as per the supplier's protocol. For the fluorometric immunostaining, the positive immunoreactions were visualized using the following fluorescent dye–conjugated secondary antibodies that were applied at room temperature for 3 hours: donkey anti-goat IgG conjugated with Alexa Fluor 488, donkey anti-rabbit IgG conjugated with Alexa Fluor 488, 594, or 633,

and donkey anti-rat IgG conjugated with Alexa Fluor 594. These secondary antibodies were used at 1:500 dilutions and were obtained from Life Technologies Corporation (Carlsbad, CA). Images were captured by a microscopy system (BX 51; Olympus, Tokyo, Japan) connected to a digital camera (DP70; Olympus) and TCS SP5 confocal system (Leica, Heidelberg, Germany), and were processed using Photoshop software version 7.0 (Adobe, San Jose, CA).

Zymography

Under deep anesthesia, brains were removed and were immediately snap frozen into liquid nitrogen. Frozen brains were serially cut with 30 μ m intervals in the coronal plane to obtain a representative cut surface of the tumor using a Leica CM3000 cryostat (Leica Microsystems, Wetzlar, Germany). Then, the surrounding nonneoplastic tissues were manually trimmed off the frozen brain; at this step, we saved OPC-rich area in tumor side. Then, six of the frozen sections (30 μ m thick) were cut from each mouse, and lysed in the sample buffer of Gelatin-Zymography Kit (Cosmo Bio, Tokyo, Japan). Protein concentration was measured by OD 280 nm for ensuring equal protein volume loading. Samples were sonicated to reduce viscosity, and electrophoresis and coomassie brilliant blue were performed according to the

Figure 2 Histological characterization of platelet-derived growth factor (PDGF)-BB-overexpressing glioma and control glioma after implantation in the brain of wild-type mouse. **A and B:** Hematoxylin and eosin (H&E) staining (**A**) and immunohistochemistry (**B**) for Olig2 of wild-type mouse brain at day 20 after the implantation of GL261-PDGF-BB and GL261-vector. **C:** Tumor volume (mm³) of GL261-PDGF-BB and GL261-vector at day 20 after implantation. **D:** Numbers of Olig2-positive cells per field in the marginal region of tumor. **E:** Immunohistochemistry for PDGF receptor α (PDGFR α) in the marginal region of tumors of GL261-PDGF-BB and GL261-vector. The images are from adjacent sections of **boxed area** in **A** and **B**. **F:** Percentage of PDGFR α -positive area per field in the marginal region of tumor of GL261-PDGF-BB and GL261-vector. **G:** Numbers of extravasated red blood cells (RBCs) along the entire circle of tumor of GL261-PDGF-BB and GL261-vector. Counted numbers of RBCs were divided by the circumferential distance of each tumor for the comparison. **H:** Higher magnification of **boxed area** of H&E staining in the marginal region of tumors of GL261-PDGF-BB and GL261-vector. **Dotted lines** in **E** and **H** indicate the edge of tumor. Error bars represent SEM (**C**, **D**, **F**, and **G**). $n = 16$ from four clones (**C**, GL261-PDGF-BB); $n = 14$ from four clones (**C**, GL261-vector); $n = 8$, two fields from four mice (**D** and **F**); $n = 4$ mice (**G**). * $P < 0.05$, *** $P < 0.001$. Scale bar: 500 μ m (**A**); 100 μ m (**E** and **H**).

manufacturer's procedure. Translucent MMP enzymatic reaction bands were measured by ImageJ software version 1.47 (NIH, Bethesda, MD; <http://imagej.nih.gov/ij/>), and normalized to the major protein bands of coomassie brilliant blue.

Image Analysis and Quantification

Images were obtained by a microscopy system (BX 51; Olympus) connected to a digital camera (DP70; Olympus).

Morphological and morphometric analyses were performed using a BioRevo BZ-9000 microscope (Keyence, Osaka, Japan), BZ-II Analyzer software version 1.41 (Keyence), and ImageJ software. The tumor volume (mm^3) was estimated on the basis of the tissue section prepared from largest cut surface of each tumor using the following equation: $\text{volume} = 4/3 \times \pi \times \text{long axis} \times \text{short axis}^2$.²⁶

Statistical Analysis

Statistical significance was determined using a *t*-test. $P < 0.05$ was considered statistically significant. Quantified data are presented as means $\pm$ SEM. Graphs were drawn using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA).

Results

Characterization of PDGF-BB–Overexpressing Glioma Cell Line

Glioma cell lines transfected with either PDGF-BB–overexpressing vector (GL261-PDGF-BB) or control vector (GL261-vector) were characterized *in vitro* before using in transplantation into mouse brain. In real-time PCR analyses, four of the GL261-PDGF-BB cell lines expressed higher levels of *Pdgfrb* mRNA compared with four of the GL261-vector cell lines (Figure 1A). The levels of *Pdgfra* mRNA expression were lower in GL261-PDGF-BB cell lines than in GL261-vector cell lines (Figure 1B). *Pdgfrb* expression was low at comparable levels in all examined clones, except that one GL261-vector cell line showed *Pdgfrb* mRNA at an exceptionally high level (Figure 1C). GL261-PDGF-BB clones released significant amounts of PDGF-BB into culture media in enzyme-linked immunosorbent assay (data not shown). In Western blot analyses, PDGFR α protein expression levels were lower in GL261-PDGF-BB than in GL261-vector cell lines (Figure 1D). The expression of PDGFR β was undetectable in all cell lines (Figure 1D). The colony-forming activity in GL261-vector cell lines was significantly higher than that in GL261-PDGF-BB cell lines ($P < 0.001$, four to five wells from each of the four clones in GL261-PDGF-BB and GL261-vector cell lines, respectively; *t*-test) (Figure 1, E and F).

PDGF-BB–Overexpressing Glioma Induces Massive Recruitment of OPCs and Exacerbates Glioma-Related Hemorrhage

First, we implanted four clones of glioma cells from lines with and without PDGF-BB overexpression into the striatum of wild-type mouse. The tumor volumes after transplantation were significantly larger in GL261-PDGF-BB than in GL261-vector (Figure 2, A–C). Olig2 is a marker of oligodendrocyte lineage, in which lineage, PDGFR α -positive cells correspond to OPCs.⁸ At higher magnification, we found that many Olig2 and PDGFR α -positive OPCs recruited and surrounded the tumor mass in all of GL261-PDGF-BB glioma, but none of GL261-vector glioma (Figure 2, A, B, and D–F). Furthermore, we found frequent hemorrhages around the tumor mass of GL261-PDGF-BB, but not GL261-vector (Figure 2H). The hemorrhagic foci were closely distributed to the recruited OPCs. The number of extravasated red blood cells around tumor was substantially larger in GL261-PDGF-BB than in GL261-vector (Figure 2G).

Pdgfra Inactivation Suppresses OPC Recruitment, Hemorrhage, and Vascular Permeability

To understand the relevance of PDGFR α signaling in the induction of cancer stroma, we next implanted GL261-PDGF-BB and GL261-vector clones into the striatum of PDGFR α -KO and Flox mice. The tumor volumes after implantation of GL261-PDGF-BB were similar between two genotypes, and *Pdgfra* inactivation in host tissue did not significantly affect the growth of implants ($P = 0.146$) (Figure 3, A, B, and G). The tumor volumes of GL261-vector were also equivalent between the two genotypes of mice (Flox, $5.06 \pm 1.60 \text{ mm}^3$; PDGFR α -KO, $8.02 \pm 1.91 \text{ mm}^3$; $P = 0.276$, data not shown). After GL261-PDGF-BB implantation, we detected many recruited OPCs and hemorrhagic foci around the tumor of Flox mice (Figure 3, A, C, D, and H–J), as previously observed after implantation in wild-type mice (Figure 2, A and D–H). In contrast, the recruitment of either Olig2-positive cells of oligodendrocyte lineage or PDGFR α -positive OPCs was largely suppressed around the tumor in PDGFR α -KO (Figure 3, B, C, H, and I). In addition, hemorrhage represented by

Figure 3 Histological characterization of platelet-derived growth factor (PDGF)-BB–overexpressing glioma (GL261-PDGF-BB) 20 days after implantation in the brain of PDGF receptor α (PDGFR α) knockout mouse (PDGFR α -KO) and PDGFR α -preserving control (Flox) mice. **A** and **B**: Hematoxylin and eosin (H&E) staining and immunohistochemistry for Olig2 of GL261-PDGF-BB in PDGFR α -KO and Flox mice. **C**: Immunohistochemistry for PDGFR α in the marginal region of tumor in Flox and PDGFR α -KO mice. The images are from adjacent sections of boxed area in **A** and **B**. **D**: H&E staining in the marginal region of tumors in Flox and PDGFR α -KO. The images are enlarged boxed area in **A** and **B**. **Insets** show the hemorrhage at higher magnification. **E**: Immunohistochemistry for IgG in Flox and PDGFR α -KO mice. **F**: Higher-magnification images of immunohistochemistry for IgG and Olig2 in the marginal region of tumor in Flox and PDGFR α -KO mice. IgG images correspond to the boxed areas in **E**, respectively. Olig2 images are from adjacent sections of boxed areas in **E**, respectively. **G**: Tumor volume (mm^3) of GL261-PDGF-BB in Flox and PDGFR α -KO (α -KO). **H**: Number of Olig2-positive cells per field in the marginal region of tumor. **I**: Percentage of PDGFR α -positive area per field in the marginal region of tumor. **J**: Numbers of extravasated red blood cells (RBCs) along the entire circle of each tumor. Counted numbers of RBCs were divided by the circumferential distance of each tumor for the comparison. **K**: Percentage of PDGFR α -positive area per field in the marginal region of tumor. Dotted lines in **C**, **D**, and **F** indicate the edge of tumor. Error bars represent SEM (**G**–**K**). $n = 4$ mice (**G** and **J**); $n = 8$, two fields from four mice (**H**, **I**, and **K**). $*P < 0.05$, $***P < 0.001$. Scale bar: 500 μm (**A**, **B**, and **E**); 100 μm (**C**, **D**, and **F**). Original magnification: $\times 400$ (**D**, **insets**, and **F**).

extravasated red blood cells in the marginal region of tumor was significantly suppressed in PDGFR α -KO (Figure 3, D and J). We examined the leakage of serum protein using immunostaining for IgG. IgG-positive area

was observed in the marginal region of tumor, corresponding to the regions where many Olig2-positive cells recruited, and the staining was more intense in Flox than in PDGFR α -KO (Figure 3, E, F, and K).

Glioma-Associated OPC Induces Vessel Dilatation and Permeability and Down-Regulates Claudin-5

The diameter of blood vessel within GL261-PDGF-BB glioma appeared to be larger in Flox than in PDGFR α -KO mice (Figure 3, A and B). Morphometrically, the diameter of CD31-positive blood vessels was significantly decreased in PDGFR α -KO compared with Flox (Figure 4, A and B). The density of the blood vessel was equivalent between Flox and PDGFR α -KO (Figure 4, A and C). When we focus on the blood vessels in the margin but not inside of the tumor, the immunofluorescence of claudin-5, a tight junction complex protein that maintains the blood tumor barrier,²⁷ was less strong in Flox than in PDGFR α -KO (Figure 4, A and D).

Glioma-Associated OPC Induces Remodeling of Collagen

Collagen type IV is one of the major components of the extracellular membrane in glioma, and is mainly synthesized by vascular endothelial cells.^{28,29} Collagen type IV expression was increased in the marginal region of GL261-PDGF-BB glioma, where many OPCs were recruited, but was low inside of the glioma in Flox mouse (some data not shown) (Figure 4, E and G). Collagen type IV expression was at low level both outside and inside of glioma in PDGFR α -KO (Figure 4, E and G). In the tumor border of Flox, but not PDGFR α -KO, triple staining for PDGFR β /collagen type IV/CD31 revealed that the collagen type IV-positive basement membrane was aberrantly thick and multilayered (Figure 4, F and H), and that the PDGFR β -positive pericytes were detached from endothelial cells and were interspersed within multilayer basement membrane (Figure 4, F and I). Next, we examined the expression of glioma invasion-related protein, MMP-9.⁴ Many recruited OPCs were highly immunoreactive for MMP-9 around tumor of Flox, but not in PDGFR α -KO, whereas glioma cells were similarly immunoreactive to MMP-9 in both genotypes of mice (Figure 4, J and K). We confirmed that most of the Sox10-positive OPCs expressed MMP-9 (Figure 4L). The pro-MMP-9 activity tended to be higher in Flox compared with PDGFR α -KO in zymography (Figure 4M), whereas the difference was not significant ($P = 0.3349$, $n = 4$ in Flox and

$n = 3$ in PDGFR α -KO mice). The pro-MMP-2 activity was similar between Flox and PDGFR α -KO.

Discussion

Herein, we implanted GL261-vector and GL261-PDGF-BB glioma cells into the brain of wild-type mouse, and found that the volume of GL261-PDGF-BB glioma was extensively larger than that of GL261-vector glioma. The large tumor in GL261-PDGF-BB was accompanied by many Olig2/PDGFR α -positive OPCs, and showed increased hemorrhage. Therefore, PDGF-BB-overexpressing glioma cells showed more high-grade malignant features, and these findings were in accordance with a previous report showing that the strength of PDGFR signaling is well correlated with high-grade characteristics of glioma.³⁰ Previously, the host tissue-derived PDGF-CC was shown to recruit OPCs toward orthotopic GL261 glioma by inactivating host *Pdgfc* or *Pdgfra* genes.³¹ In our present study, an inactivation of host *Pdgfra* resulted in the substantial decrease of OPC recruitment in GL261-PDGF-BB glioma, and it was clearly shown that glioma-derived paracrine signal of PDGF-BB recruited OPCs via PDGFR α activation in OPCs. Intriguingly, the inactivation of host *Pdgfra* was accompanied by the decrease of hemorrhage and vascular leakage in PDGFR α -KO mouse, even though the glioma volume was comparable between Flox and PDGFR α -KO mice. It was suggested that the paracrine PDGF signal of glioma could be causally related with hemorrhage and brain edema that contribute to the serious prognostic factors of GBM, independently of the tumor volume.²

Activated PDGFR α after intracerebral hemorrhage induced blood-brain barrier (BBB) dysfunction in ischemic stroke and cerebral hemorrhage, in which MMP-9 was critically involved.^{9,32} Similarly, OPCs were shown to release MMP-9 under acute phase of white matter injury to initiate the early onset of BBB breakdown.³³ We found frequent hemorrhage and increased IgG leakage in the marginal region of GL261-PDGF-BB glioma, where MMP-9-expressing OPCs were recruited. Specific to this area, aberrant vasculatures were found that were accompanied by a multilayered thick collagen; they contain interspersed pericytes, whose vascular phenotypes were

Figure 4 Morphological analyses of the parameters related with blood vessel formation and extracellular matrix in platelet-derived growth factor (PDGF)-BB-overexpressing glioma. **A:** Immunohistochemistry for CD31 and immunofluorescence for claudin-5 (red) in the marginal region of tumor of Flox and PDGFR α -KO. **B and C:** Blood vessel diameter and numbers measured from the images of CD31 staining, respectively. **D:** Claudin-5 immunofluorescence intensity per blood vessel was converted into gray values, and mean gray value per blood vessel was calculated. **E:** Immunohistochemistry for collagen type IV in the border part of tumor of Flox and PDGFR α -KO mice. **F:** Triple immunofluorescence staining for PDGFR α (green), collagen type IV (magenta), and CD31 (red) in the border part of tumor of Flox and PDGFR α -KO. **Boxed areas in the left panels** are shown at higher magnification in the **right panels**. **G:** Percentage of collagen type IV-positive area per field. **H:** Thickness of the collagen type IV-positive basement membrane of blood vessel. **I:** Measurement of stratification of the PDGFR β -positive cells around the blood vessels. **J:** Immunohistochemistry for matrix metalloproteinase (MMP)-9 in the marginal region of the tumor of PDGFR α -KO and Flox mouse. **K:** Number of MMP-9-positive cells in the marginal region per field. **L:** Double-immunofluorescence staining for SOX10 (green) and MMP-9 (red) in the marginal region of the tumor of PDGFR α -KO and Flox mice. **M:** A representative image of the gelatin zymography gel. **Dotted lines in A, J, and L** indicate the edge of tumor. Error bars represent SEM (**B–D, G–I, and K**). $n = 16$, each four fields from four mice (**B and C**); $n = 77$ (**D**, blood vessels from nine Flox mice); $n = 83$ (**D**, blood vessels from eight PDGFR α -KO mice); $n = 8$, two fields each from four mice (**G and K**). *** $P < 0.001$. Scale bars: 100 μ m (**A**, CD31); 50 μ m [**A**, claudin-5, **E, F** (**left panels**), **J**, and **L**]; 20 μ m [**F** (**right panels**)].

similar to those reported in previous experimentally induced glioma.^{34,35} These vascular abnormalities in the tumor border of GL261-PDGF-BB in Flox mice were mostly normalized in PDGFR α -KO. Taken altogether into consideration, recruited OPCs by paracrine PDGF signal were indicated to be involved in the BBB dysfunction to cause hemorrhage and vascular leakage. Decreased expression of claudin-5, a tight junction complex protein, that maintains the blood-tumor barrier,²⁷ could be an underlying cause of BBB dysfunction; however, the mechanism of decrease remains unknown. Glioma cell–derived MMP-9 was not sufficient to induce hemorrhage within tumor, suggesting that OPC-derived additional factors were required for the vascular phenotype.

MMPs strongly correlate with malignant progression of glioma,^{36,37} among which MMP-9 mediates glioma cell adhesion to the extracellular membrane and invasive growth along collagen type IV.³⁸ In our study, a cancer stroma was established with abundant collagen type IV and MMP-9 expression around GL261-PDGF-BB glioma in wild-type and Flox mice, but not in that of PDGFR α -KO mice; however, the tumor volume was similar between *Pdgfra*-preserving mice and PDGFR α -KO mice. Different PDGF signal in glioma may differentially regulate invasive tumor growth and associating events, like hemorrhage and edema; further studies are needed.

Colony-forming activities were significantly lower in GL261-PDGF-BB than in GL261-vector *in vitro*, which was compatible with low expression levels of PDGFRs in GL261-PDGF-BB glioma cells. The volume of either GL261-PDGF-BB or GL261-vector glioma was comparable between Flox and PDGFR α -KO mice. Accordingly, either of autocrine PDGF-BB signal in glioma or host PDGFR α signaling was not a major determinant of GL261 glioma volume in our study. These observations implied that activated host PDGFR β signaling might have been involved in the larger GL261-PDGF-BB than GL261-vector in wild-type mice. One of the subclasses of GBM was characterized by high levels of PDGF-B ligand expression and PDGFR β phosphorylation.¹² The PDGFR β was insignificant in our GL261 cells. Further studies are required to explore the contribution of the two types of PDGFRs in PDGF-BB–overexpressing glioma.

Our study showed that glioma-derived paracrine signal of PDGF-B extensively recruits OPCs via the activation of PDGFR α , and that the recruited OPCs were implicated in the formation of cancer stroma that defines microenvironment, such as BBB dysfunction, hemorrhages, and vascular abnormality with extracellular membrane remodeling. PDGFR α inactivation could normalize vascular phenotype induced by PDGF-BB secreted from glioma, and vascular normalization is crucial for radiation therapy of GBM.³⁴ Our findings may open prospects to develop a new therapeutic target for GBM and other hypervascular brain tumors.

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