The Contribution of Bone Marrow–Derived Cells to the Tumor Vasculature in Neuroblastoma Is Matrix Metalloproteinase-9 Dependent

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Abstract

The contribution of the tumor stroma to cancer progression has been increasingly recognized. We had previously shown that in human neuroblastoma tumors orthotopically implanted in immunodeficient mice, stromal-derived matrix metalloproteinase-9 (MMP-9) contributes to the formation of a mature vasculature by promoting pericyte recruitment along endothelial cells. Here we show that MMP-9 is predominantly expressed by bone marrow–derived CD45-positive leukocytes. Using a series of bone marrow transplantation experiments in MMP-9+/− and MMP-9−/− mice xenotransplanted with human neuroblastoma tumors, we show that bone marrow–derived MMP-9 is critical for the recruitment of leukocytes from bone marrow into the tumor stroma and for the integration of bone marrow–derived endothelial cells into the tumor vasculature. Expression of MMP-9 by bone marrow–derived cells in the tumor stroma is also critical for the formation of a mature vasculature and coverage of endothelial cells with pericytes. Furthermore, in primary human neuroblastoma tumor specimens of unfavorable histology, we observed a higher level of tumor infiltration with MMP-9 expressing phagocytic cells and a higher degree of coverage of endothelial cells by pericytes when compared with tumor specimens with a favorable histology. Taken together, the data show that in human neuroblastoma tumors orthotopically implanted neuroblastoma tumors, MMP-9 contributes to tumor angiogenesis by promoting the recruitment of pericytes along vascular endothelial cells (15). MMP-9 also plays a role in the mobilization of hematopoietic stem cells into the circulation. In mice treated with the myelosuppressive agent 5-fluourouracil (5-FU), MMP-9 promotes the release of soluble c-kit ligand and permits the transfer of hematopoietic and endothelial stem cells from a quiescent to a proliferative compartment in the bone marrow (16). This observation raises the possibility that MMP-9 could also contribute to tumor progression by increasing the presence of bone marrow–derived precursor cells available for recruitment in the tumor stroma.

Childhood neuroblastoma is the second most common extracranial tumor in children (17). Based on their histopathology, neuroblastoma tumors are classified as having a favorable or unfavorable histology. Tumors with a favorable histology typically contain an abundant Schwannian stroma have a low mitosis-karyorrhexis index, are not MYCN amplified, are seen in younger children, and have a favorable clinical outcome. In contrast, tumors with an unfavorable histology are typically characterized by a paucity of stroma, contain few or no Schwann cells, have a high mitosis-karyorrhexis index, are MYCN amplified, and are associated with a poor clinical outcome (18, 19).

We have previously reported the presence of higher levels of stromal-derived MMP-9 in primary human neuroblastoma tumor specimens from patients with advanced stage (III and IV) disease when compared with specimens from patients with localized disease (stage I or II; ref. 20). More recently, we have shown that in MMP-9–deficient mice orthotopically implanted with SK-N-BE (2) human neuroblastoma there is a defect in the maturation
of the tumor vasculature characterized by the presence of small vessels and poor pericyte coverage (15). Here we have examined the stromal origin of MMP-9 expression in these tumors and have observed that MMP-9 is predominantly expressed by CD45-positive bone marrow–derived leukocytes. This observation led us to conduct a series of bone marrow transplantation experiments in mice implanted with SK-N-BE (2) neuroblastoma tumors to determine the contribution of bone marrow–derived MMP-9 to tumor angiogenesis and vasculogenesis.

Materials and Methods

Cell culture. The human neuroblastoma cell line SK-N-BE (2) was obtained from Dr. J. Biedler (Memorial Sloan-Kettering Cancer Center, New York, NY). Cells were routinely grown in RPMI (Cellgro, Mediatech, Inc., Herndon, VA) supplemented with 10% fetal bovine serum (Omega Scientific, Inc., Tarzana, CA), penicillin-streptomycin (1%), and 2 mmol/L L-glutamine (Irvine Scientific, Santa Ana, CA).

Bone marrow transplantation experiment. Eight-week-old mice in the FVB/N background carrying homozygous null mutations in the Rag1 gene and the MMP-9 gene as previously reported (15) were used in all experiments. The immunodeficient status of the mice was verified by fluorescence-activated cell sorting (FACS) analysis (Becton Dickinson, San Jose, CA) of peripheral blood for the absence of T and B lymphocytes. Animals were maintained in a germ-free barrier facility and procedures were done according to the guidelines of the Institutional Animal Care Utilization Committee at the Childrens Hospital Los Angeles (Los Angeles, CA). Mice were arranged into four experimental groups for the performance of syngeneic age-matched bone marrow transplantation using MMP-9+/+ (RAG1<sup>ko/ko</sup>) and MMP-9<sup>−/−</sup> (RAG1<sup>ko/ko</sup>/MMP-9<sup>g<sup>ko/ko</sup></sup>) donor and recipient combinations (Table 1). Male mice were used as bone marrow donors and were treated with 150 mg/kg of 5-FU (American Pharmaceutical Partners, Inc., Los Angeles, CA) given i.p. 2 days before sacrifice and bone marrow harvest. Bone marrow cells were harvested from both femurs and tibias by flushing the bone cavity 2 days before sacrifice and bone marrow harvest. Bone marrow cells with basal bone marrow medium (BBMM); Iscove’s medium (Cambrex, Walkersville, MD), supplemented with 30% fetal bovine serum (Stem Cell Technologies, Vancouver, British Columbia, Canada), 1% bovine serum albumin (Sigma, St. Louis, MO), 100 mmol/L 2-mercaptoethanol (Sigma), 2 mmol/L L-glutamine, 1% penicillin/streptomycin, and 1% fungione (Irvine Scientific). After washing with PBS, bone marrow cells were resuspended at 1 to 2 × 10<sup>8</sup> nucleated cells/ml in BBMM supplemented with 50 ng/ml recombinant human interleukin 6 (R&amp;D Systems, Minneapolis, MN), 10 ng/ml murine interleukin 3, and 2.5 ng/ml murine stem cell factor (BioSource International, Camarillo, CA; BBMM-GF). The bone marrow cells were transduced with a retroviral vector containing the cDNA of the enhanced green fluorescent protein (EGFP). In brief, GPE packaging cells containing the MM-EGFP-SAR retroviral vector (obtained from Dr. Donald Kohn, Childrens Hospital Los Angeles) were irradiated at 4,000 cGy once with a Gammacell 1000 Cesium-137 cell irradiator (Atomic Energy of Canada Ltd., Mississauga, Ontario, Canada) and plated at 1 × 10<sup>6</sup> cells per 100-mm dish in DMEM (Cellgro, Mediatech) containing 10% newborn calf serum (Cellgro). After 24 hours, 1 to 2 × 10<sup>7</sup> bone marrow cells were added to the packaging cells and incubated for 72 hours in BBM-GF containing 4 mg/ml polybrene (Sigma). On the day of transplantation, nonadherent and adherent cells were collected, washed thrice with PBS, and counted in an hemocytometer. Cells were injected at 3.5 × 10<sup>5</sup> mouse iv. into female recipient mice that had been irradiated at 300 cGy on two consecutive days using a MK-1-68A Cesium-137 Gamma animal irradiator (J.L. Shepherd and Associates, San Fernando, CA).Recipient mice were maintained in a germ-free environment and treated with oxymetacrine (Phoenix Pharmaceutical, Inc., St. Joseph, MO) added at 200 μg/ml to the drinking water for the entire duration of the experiment.

Xenotransplanted orthotopic neuroblastoma model and tissue harvest. After full bone marrow engraftment (day >30 after bone marrow transplant) recipient mice were implanted with SK-N-BE (2) human neuroblastoma tumors sown onto the left adrenal gland as previously reported (15, 21). Three weeks after tumor implantation, mice were anesthetized with 2% Avertin, the chest was opened, and the vasculature was washed free of blood by slow perfusion of 20 ml of 0.9% NaCl into the left ventricle. Tumors were dissected from the kidneys and their volume determined with a caliper using the formula (π/6 × L × T)^2/2.

Peripheral blood count. Twenty microliters of peripheral blood were collected from the tail vein of each mouse and WBCs were manually counted in a hemocytometer as per manufacturer’s instructions (Unopette Microcollection System, Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ).

FACS analysis of the peripheral blood nucleated WBC. Peripheral blood samples were incubated with BSC lysis buffer (156 mmol/L NH<sub>4</sub>C1, 0.1 mmol/L EDTA, and 12 mmol/L NaHCO<sub>3</sub>) for 10 minutes. WBC were washed and resuspended in PBS. The percentage of EGFP-labeled WBC was quantified by FACS analysis using a FACS caliber immunocytochemistry equipped with the Cellquest software (Becton Dickinson). For the determination of WBC subclasses, cells were incubated with antibodies against mouse CD4 and CD8 (T lymphocytes), CD19 (B lymphocytes), Gr-1PE (granulocytes), CD11b (macrophages), and Dx5 (natural killer cells; PharMingen, San Jose, CA) for 30 minutes before being examined by FACS analysis.

Angiography. Biotinylated Lycopersicon esculentum (tomato) lectin (50 μL) and Texas red avidin D (100 μL; Vector Laboratories, Inc., Burlingame, CA) were mixed for 20 minutes before iv. injection in tumor bearing mice (150 μL/mouse). Five minutes after injection, animals were anesthetized with 2% Avertin, perfused with 0.9% NaCl as described above and the tumors were harvested and examined unstained or under fluorescent confocal microscopy using a Leica SP confocal DM IRBE-inverted microscope equipped with a 488-nm argon ion laser and BSP500 beam splitter as previously described (15).

SDS-PAGE. Frozen tumor tissues were homogenized in lysis buffer [10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 10% Glycerol, 1 Triton X-100]. Aliquots of tumor lysates containing 20 μg of proteins were loaded on 0.1% SDS, 8% polyacrylamide gels containing 1% gelatin. After electrophoresis, gels were incubated overnight at 37°C in substrate buffer [50 mmol/L Tris-HCl (pH 7.4) and 10 mmol/L CaCl<sub>2</sub>], stained with 0.25% Coomassie brilliant blue, and destained in methanol/acetic acid/water (50:10:80). Aliquots of serum-free conditioned medium from 12-O-tetradecanoylphorbol-13-acetate–treated human HT1080 cells and recombinant murine MMP-9 (R&amp;D Systems) were used as markers for human and murine MMP-9, respectively.

Immunofluorescence. Primary antibodies used for immunofluorescence were a goat polyclonal antibody against CD51/platelet-endothelial cell adhesion molecule 1 (PECAM-1) for endothelial cells (Santa Cruz Biotechnology, Santa Cruz, CA), a mouse monoclonal anti-smooth muscle actin (SMA) antibody for pericytes (DakoCytomation, Carpinteria, CA), a rabbit polyclonal anti-EFPP antibody (Novus Biologicals, Littleton, CO), goat anti-mouse and goat anti-human MMP-9 antibodies (R&amp;D Systems), a mouse anti-mouse CD45.1/Ly-5.1 monoclonal antibody for leukocyte detection (SouthernBiotech, Birmingham, AL), a rabbit polyclonal antibody against human lysozyme for phagocytic cells.

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detection (EC 3.2.1.17 from DakoCytomation; ref. 22), and a rabbit anti-human S100B antibody for Schwann cells (DakoCytomation). Cy3 or FITC-conjugated anti-IgG secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were selected according to the primary antibody. Five-micrometer paraffin-embedded sections of tumors or organs were deparaffinized, microwave in antigen unmasking solution (Vector Laboratories), and incubated with the primary antibody overnight at 4°C. The secondary antibody was applied for 45 minutes at room temperature. Sections were mounted with 6/25-diamino-2-phenylindole-containing medium (Vector Laboratories) and viewed with a Leica DMRA microscope (Wetzlar, Germany) using Plan Apo 20×/1.6 NA phase differential interference contrast microscope or Plan Apo 100×/1.6 oil immersion objective lenses. The microscope was equipped with a Sutter LS175W ozone-free xenon arc lamp. Images were acquired with an Applied Spectral Imaging Ltd. SkyVision-2/VDS camera from Easy fluorescence in situ hybridization software (Migdale Ha’Emek, Israel). Quantitative analysis was done using MetaMorph 6.2 software. Vessel maturation was quantified by calculating the ratio of pericyte area/endothelial cell area.

Human neuroblastoma tumor samples. Paraffin-embedded sections of archival primary adrenal and paraspinal human neuroblastoma tumor samples were obtained by Dr. H. Shimada and assigned into favorable histology and unfavorable histology groups according to their histopathology (19). Twelve human cases (7 favorable histology tumors and 5 unfavorable histology tumors) were examined. All the favorable histology cases had nonamplified MYCN gene and included three ganglioneuroblastoma (Schwannian stroma rich; diagnosed at the age of 1 year 10 months, 3 years 2 months, and 14 years 6 months, respectively), and four tumors showing an appearance of neuroblastoma (Schwannian stroma poor), poorly differentiated subtype with a low mitosis-karyorrhexis index (diagnosed at the age of 28 days, 2, 5, and 6 months, respectively). Two unfavorable histology tumors showed the same appearance of poorly differentiated neuroblastoma with a low mitosis-karyorrhexis index (diagnosed at the age of 4 years 8 months and 10 years 7 months) and had nonamplified MYCN gene. The three other unfavorable histology tumors had amplified MYCN gene: two of them were neuroblastoma, undifferentiated subtype with a high mitosis-karyorrhexis index (diagnosed at the age of 1 year and 6 months and 4 years and 4 months), and one was neuroblastoma, poorly differentiated subtype with an intermediate mitosis-karyorrhexis index (1 year and 6 months of age at the time of diagnosis). All these unfavorable histology tumors were classified as Schwannian stroma poor. Specimens were randomly coded and blindly analyzed. The study was approved by the Committee of Clinical Investigations at Children’s Hospital Los Angeles.

Statistical analysis. The two-sample t test was used in all experiments for comparison of the average values among four bone marrow transplant groups.

Results

Expression of matrix metalloproteinase-9 in orthotopically xenotransplanted neuroblastoma tumors. To better define the cellular source of stromal expression of MMP-9 in xenotransplanted tumors, we did dual immunofluorescence analysis on sections of SK-N-BE (2) neuroblastoma tumors orthotopically implanted in RAG-1-/- mice. In these tumors, large nests of tumor cells were separated by connective tissue septa that could be easily identified on H&E-stained sections (Fig. 1A). MMP-9 expressing cells were identified along CD31/PECAM expressing endothelial cells (Fig. 1B) and SMA (Fig. 1C) expressing pericytes but neither endothelial cells nor pericytes showed MMP-9 expression. In contrast, staining for MMP-9 and CD45, an epitope present on the surface of leukocytes, including lymphocytes, polymorphonuclears, and monocytes, indicated that these cells were the predominant source of MMP-9 (Fig. 1D).

(Note that because mice were RAG-1-/-, lymphocytes are absent.) The expression of MMP-9 by CD45-positive leukocytes thus suggested that transplantation of tumor bearing MMP-9+/+ recipient mice with bone marrow cells from MMP-9 expressing donor mice may correct the defect in the tumor vasculature. Transplantation of MMP-9+/+ bone marrow cells corrects the vascular defect in tumors of MMP-9-/- mice. To test this hypothesis, we did a series of bone marrow transplantation experiments in MMP-9+/+ and MMP-9-/- mice that were orthotopically implanted with SK-N-BE (2) neuroblastoma tumors after successful bone marrow engraftment. We used MMP-9+/+ and MMP-9-/- donor and recipient combinations to generate four experimental groups (Table 1). To monitor engraftment and the fate of these cells post-transplantation, harvested donor bone marrow cells were transduced in vitro with an EGFP expressing retroviral vector before being injected into subletally irradiated (500 cGy daily × 2) mice. After successful engraftment (4 weeks), recipient mice were orthotopically implanted with SK-N-BE (2) neuroblastoma tumors. Three weeks after tumor implantation, in vivo fluorescence angiography was done and tumors were harvested. Analysis of the tumor vasculature by fluorescence angiography indicated the presence of a well-developed network of vessels in tumors derived from group 1 and 2 mice (transplanted with bone marrow cells from MMP-9+/+ donor mice) when compared with tumors derived from group 3 and 4 mice (transplanted with MMP-9+/+ donor bone marrow cells), which consistently exhibited a poorly developed vasculature with small blood vessels (Fig. 2A).

Next, we examined the level of pericyte coverage of endothelial cells in these tumors by double immunofluorescence histology using CD31/PECAM as a marker for endothelial cells and SMA as a marker for pericytes (Fig. 2B). This analysis revealed an anticipated significant lack of pericyte coverage in tumors derived...
from MMP-9−/− recipient mice transplanted with bone marrow cells from MMP-9−/− donor mice (group 4) which had a pericyte area of 1,120 ± 407 μm² when compared with tumors derived from MMP-9+/+ recipient mice transplanted with bone marrow cells from MMP-9+/+ donor mice (group 1) which had a pericyte area of 4,808 ± 1,027 μm² (P < 0.001). These data are consistent with our previously published observations (15). Tumors derived from group 4 mice also had significantly lower values of endothelial cell area (618 ± 233 μm²) and exhibited a lower pericyte/endothelial cell ratio (1.98 ± 0.7) compared with tumors derived from group 1 (3.95 ± 1.08; P < 0.001). Restoration of pericyte coverage was achieved by transplanting MMP-9+/+ recipient mice with bone marrow cells from MMP-9+/+ donor mice (group 2) as tumors from this group exhibited pericyte area and endothelial cell area values (5,060 ± 596 and 1,325 ± 154 μm²) and a pericyte/endothelial cells ratio (4.5 ± 0.77) similar to values observed in tumors from group 1 (P = 0.4; Fig. 2C). In these tumors, vessels were well developed with multiple layers of pericytes surrounding endothelial cells as observed in tumors derived from group 1 mice. In contrast, in tumors derived from MMP-9+/+ recipient mice transplanted with bone marrow cells from MMP-9+/+ donor mice (group 3), significant lower values for pericyte and endothelial cell areas were observed (1,433 ± 377 and 673 ± 252 μm² respectively; P < 0.001) and the pericyte/endothelial cell ratio was lower (2.37 ± 0.72; P = 0.03) when compared with group 1 or 2 tumors. These differences in pericyte coverage among the four experimental groups were not observed in the vasculature of normal organs like muscle, liver, lungs, and kidney (data not shown), which points to a more specific role for MMP-9 in pathologic rather than normal angiogenesis. The lack of pericyte recruitment in tumors derived from group 3 mice when compared with tumors derived from group 2 mice suggests that bone marrow–derived cells are the major source of MMP-9 in the tumor stroma and that cells from neighboring tissue do not substantially contribute to MMP-9 expression. To confirm this possibility, we did an analysis of MMP-9 expression in tumor lysates by gelatin zymography (Fig. 3A). All tumors in group 1 and 2 mice showed, as anticipated, the presence of a M, 95,000 gelatinolytic band that comigrated with recombinant murine proMMP-9. This band was not detected in tumors derived from group 4 mice. Whereas the majority of tumors derived from group 3 mice were negative for MMP-9, some tumors contained low levels of murine proMMP-9, an observation that suggested that bone marrow cells may not be the only source of MMP-9, because these mice were transplanted with MMP-9−/− bone marrow cells. However, an immunohistologic analysis of the bone marrow of these mice revealed the presence of MMP-9-positive cells that did not express EGFP, indicating the presence of a mixed chimism.

Figure 2. Transplantation of MMP-9+/+ bone marrow cells corrects the vascular defect of tumors implanted in MMP-9−/− mice. A, tumor vascular architecture examined by in vivo angiography as described in Materials and Methods. Representative pictures are a reconstruction of the maximum intensity in 50 Z-series sections at 2-μm intervals obtained by confocal microscopy. Three fresh tumors in each group were examined. Bar, 200 μm. B, representative pictures of paraffin-embedded tissue sections evaluated by double immunofluorescence for pericytes (SMA, green) and endothelial cells (EC, PECAM, red). Bar, 50 μm. Insets, high magnification (100/1.6) of a vessel cross-section. C, amount of pericytes and endothelial cells in the tumor sections was quantified by measuring the amount of green and red fluorescent area as indicated in Materials and Methods in 30 histological fields for each tumor section. The level of pericyte coverage was expressed as pericyte/endothelial cell ratio and was calculated from the data above. Average area per field in each tumor generated from three independent BMT experiments. Number of tumors examined in each group: group 1, n = 5; group 2, n = 7; group 3, n = 5; and group 4, n = 6.
with the persistence of some MMP-9-positive recipient cells post-BMT (Fig. 3B). In the tumor tissue, these cells were also detected as MMP-9-positive, EGFP-negative cells that coexpressed CD45 (Fig. 3C). The presence of MMP-9 in some tumors derived from MMP-9+/+ recipient mice transplanted with bone marrow cells from MMP-9−/− donor mice is therefore attributed to the presence of a bone marrow mixed chimerism where some recipient bone marrow cells survived despite sublethal irradiation. Altogether, the data support our hypothesis that bone marrow–derived cells are the primary source of MMP-9 in xenotransplanted tumors and show that expression of MMP-9 by these cells is critical for the establishment of a mature vasculature and pericyte recruitment.

Matrix metalloproteinase-9 contributes to the recruitment of bone marrow–derived cells to the tumor microenvironment. It has recently been suggested that MMP-9 contributes to the mobilization of bone marrow stem cells into the circulation (16). We therefore asked the question whether MMP-9 could affect the recruitment of bone marrow–derived cells from the blood circulation into the tumor microenvironment. To address this question, we examined the presence of EGFP expressing cells in the stroma of tumors obtained in each experimental group (Fig. 4). This analysis indicated the presence of numerous EGFP-positive bone marrow–derived cells in the stroma of tumors derived from group 1 (19.4 ± 4.9 per field) and group 2 (19.4 ± 5 per field) recipient mice transplanted with bone marrow cells from MMP-9−/− donor mice (Fig. 4A and B). In contrast, significantly fewer EGFP-positive bone marrow–derived cells were present in tumors derived from group 3 (2.7 ± 0.6 per field) and group 4 (0.97 ± 0.7 per field) recipient mice transplanted with bone marrow cells from MMP-9−/− donor mice (P < 0.01). To determine whether these differences were due to differences in engraftment and release of bone marrow–derived cells into the circulation, we analyzed the number of EGFP-positive WBC in the peripheral blood 4 weeks after transplantation when the mice were implanted with SK-N-BE (2) tumors (Fig. 4C). The number of WBC was significantly higher in group 1 mice compared with the other three groups (8.1 mm−3, P < 0.01) but was not different between group 2 and 3 (3.35 and 2.9 mm−3, P = 0.1). There was no difference in the relative number of granulocytes, macrophages, and natural killer cells between wild-type and MMP-9−/− mice in all experimental groups. The percentage of peripheral blood WBC expressing EGFP was similar, ranging between 75% and 80% (reflecting similar levels of transduction efficiency). The difference in the number of EGFP-positive WBC between groups 1 and 2 might suggest that stromal-derived MMP-9 contributes to the mobilization of WBC from the bone marrow space to the blood circulation. However, the degree of infiltration of these cells into the tumor tissue was not different. The difference in the amount of EGFP-positive cells present in the tumor stroma between group 2 and 3 mice in the absence of a similar difference in the peripheral blood (P = 0.2) suggests that MMP-9 expression by WBC contributes to their recruitment from the peripheral blood into the tumor stroma.

Hematopoietic stem cells are a source of EPC that contribute to the tumor vasculature (6). To determine whether bone marrow–derived endothelial cells contribute to the tumor vasculature in our model and whether MMP-9 was involved in this process, we examined tumors in the four experimental groups for the presence of EGFP-positive cells also expressing CD31/PECAM (Fig. 5). By double immunofluorescence analysis, we detected CD31/PECAM and EGFP coexpressing cells in the vasculature of tumors derived from group 1 and 2 mice where they represented 13.3 ± 2% and 14 ± 3.9% of the total CD31/PECAM positivity, respectively. In contrast, no EGFP-positive cells coexpressing CD31/PECAM could be detected in group 3 and 4 tumors (Fig. 5C). To determine whether bone marrow–derived cells could also differentiate into pericytes, tumor sections were double stained for EGFP and SMA. This analysis (Fig. 5D) failed to identify EGFP-positive cells that were also SMA positive in tumors of the four experimental groups. Thus, in addition to promoting pericyte recruitment, MMP-9 also plays a role in the recruitment of bone marrow–derived endothelial cells in the tumor microenvironment where these cells contribute to vasculogenesis.

Primary neuroblastoma tumors with an unfavorable histology have higher infiltration with matrix metalloproteinase-9–expressing inflammatory cells and a more mature vasculature. From our experiments in xenotransplanted tumors, we would anticipate that in advanced-stage human neuroblastoma
tumors, there would be a higher level of infiltration with MMP-9–expressing cells and a higher level of pericyte coverage of endothelial cells. To test this hypothesis, we examined a series of primary human neuroblastoma tumors for the presence of pericytes and MMP-9–expressing cells. We analyzed five tumors with unfavorable histology and seven tumors with a favorable histology. Analysis of the pericyte/endothelial cell ratio in the tumor samples indicated the presence of more mature vessels in unfavorable histology tumors with an average pericyte/endothelial cell ratio of 3.0 ± 0.7 (Fig. 6A and C), whereas favorable histology tumors exhibited immature vessels poorly covered with pericytes as indicated by a significantly lower pericyte/endothelial cell ratio (1.8 ± 0.45; \( P = 0.01 \)). Unfavorable histology tumors had a lower microvessel density (19 ± 7.8 vessels per field) than favorable histology tumors (48 ± 11 vessels per field; \( P < 0.001 \)). However, the average tumor vessel size in unfavorable histology tumors was 2.3× larger (550 ± 296 pixels per field) than in favorable histology tumors (239 ± 90 pixels per field; \( P = 0.07 \); Fig. 6C). The endothelial cell area between these two types of tumors was however no different (906.6 versus 1,177 \( \mu \text{m}^2 \); \( P = 0.4 \)) thus indicating that for the same area of endothelial cells, unfavorable histology tumors contained larger and more mature vessels in smaller numbers than favorable histology tumors which were rich in small but immature vessels. When examined for the presence of Schwann cells with an anti-S100B antibody unfavorable histology tumors did not stain positive, whereas favorable histology tumors, and in particular ganglioneuroblastoma, stained strongly positive (Fig. 6B). When examined for MMP-9 expression, unfavorable histology tumors also contained a substantially higher number of MMP-9–expressing cells than favorable histology tumors (24 ± 8.9 versus 2.7 ± 0.9 cells per field, \( P < 0.01 \); Fig. 7A and C), and the majority of these MMP-9-positive cells contained lysozyme, indicating they were phagocytic cells, (Fig. 7B). In summary, we have shown that bone marrow–derived inflammatory cells are the primary source of MMP-9 expression in neuroblastoma tumors where MMP-9 has a dual role: it promotes pericyte coverage of endothelial cells and it is important for the recruitment of inflammatory cells to the tumor microenvironment. Both roles are associated with the more aggressive behavior and poor clinical outcome that characterize unfavorable histology tumors.

**Discussion**

The contribution of the tumor stroma as a source of MMP-9 expression in animal models of tumorigenesis and in human cancer has been well recognized. In murine models of squamous epithelial carcinoma and pancreatic cancers, MMP-9 is expressed by inflammatory cells like mast cells, neutrophils, and macrophages where it plays a critical role in initiating an angiogenic switch (13, 14). In ovarian cancer cells xenotransplanted in nude mice, MMP-9 is expressed by tumor-associated macrophages where it promotes angiogenesis and growth (23). MMP-9 expression is also specifically induced in lung endothelial cells and macrophages by distant primary tumors via VEGF receptor (VEGFR-1) and promotes the establishment of lung metastasis (24). In human cancers, like breast, colon, bladder, skin, and prostate cancers, MMP-9 is predominantly expressed by stromal fibroblasts and its expression correlates with clinical outcome (25–29). We previously reported that in both primary human neuroblastoma tumors and in orthotopically xenotransplanted tumors, MMP-9 is expressed by the stroma and that MMP-9 contributes to the tumor vasculature by promoting the coverage of endothelial cells by pericytes (15, 20). In this article, we provide three lines of evidence indicating that in neuroblastoma bone marrow–derived leukocytes are the primary source of MMP-9 expression. In our xenotransplanted orthotopic tumor model, we show that CD45-positive leukocytes (granulocytes and monocytes) express MMP-9 whereas CD31/PECAM-positive endothelial cells or SMA-positive pericytes do not express the enzyme. Second, our transplantation experiments clearly indicate that transplantation of MMP-9-positive bone marrow cells in MMP-9 null mice results in tumor colonization by MMP-9-positive leukocytes and can restore the vascular architecture. Third, in unfavorable histology human neuroblastoma tumor...
samples, MMP-9 is expressed by phagocytic cells (activated macrophages). Whereas we cannot entirely rule out some contribution of other non-bone marrow–derived cells as a source of MMP-9, our data support the concept that bone marrow–derived cells are the predominant contributor of MMP-9 expression. The fact that we observed some MMP-9 expression in the tumor tissues of neuroblastoma tumors implanted in MMP-9+/+ recipient mice transplanted with MMP-9−/− bone marrow cells (group 3) is not inconsistent with our hypothesis because we documented the presence of a bone marrow–mixed chimera in these mice.

Our data also show that the recruitment of bone marrow–derived cells into the tumor is dependent on MMP-9 expression by these cells because the number of MMP-9-positive cells that colonized neuroblastoma tumors implanted in mice was a function of the genotype of the bone marrow donor and not of the genotype of the recipient. Expression of MMP-9 by bone marrow cells releases the soluble c-kit ligand permitting the transfer of endothelial cells and hematopoietic stem cells from a quiescent to a proliferative niche in the bone marrow and to the circulation, resulting in a more rapid recovery post myeloablative therapy (30). Consistent with these data, we observed a larger number of EGFP-positive bone marrow–derived cells in the tumor stroma of MMP-9−/− mice transplanted with MMP-9+/+ bone marrow cells (group 2) than in MMP-9−/− mice transplanted with

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**Figure 5.** Presence of bone marrow–derived endothelial cells in xenotransplanted tumors. A, representative pictures of paraffin-embedded xenotransplanted tumor sections immunostained for EGFP (green) and PECAM (red). White arrowheads: presence of EGFP and PECAM coexpressing cells in the endothelium layer of blood vessels. B, representative pictures of paraffin-embedded tumor samples immunostained for EGFP (red) and SMA (green). Bars, 50 μm in all pictures. C, quantification analysis of the amount of bone marrow–derived endothelial cells incorporated into the tumor vasculature. Average percentage of PECAM and EGFP colocalization area from the total PECAM-positive area per microscopic field (vasculogenesis). Ten microscopic fields were examined in two tissue sections for each tumor. Number of tumors examined: group 1, n = 4; group 2, n = 8; group 3, n = 5; and group 4, n = 5.

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**Figure 6.** Primary neuroblastoma tumors with an unfavorable histology have better endothelial cell (EC) coverage by pericytes. A and B, representative pictures of sections of primary human neuroblastoma tumors immunostained for pericytes (green) and endothelial cells (red) in (A) and for Schwann cells (red) and pericytes (green) in (B). Bar, 50 μm. C, quantitative analysis of pericyte/endothelial cell ratio, microvessel density (MVD), microvessel size (MVS), and endothelial cell area per field in the tumors. Average values from thirty microscopic fields examined in each tumor. Number of tumors examined: unfavorable histology (UH), n = 5 and favorable histology (FH), n = 7.
MMP-9 has been shown to contribute to tumor angiogenesis in a complex way. Most of the mechanisms have pointed to endothelial cells where MMP-9 promote their ability to form tubes on Matrigel (31), trigger an angiogenic switch by increasing endothelial cells where MMP-9 promote their ability to form marrow–derived endothelial cells in the tumor vasculature. Using the solubilization of VEGF (13), or as discussed above, by tubes on Matrigel (31), trigger an angiogenic switch by increasing endothelial cells where MMP-9 promote their ability to form marrow–derived endothelial cells in the tumor vasculature. Because we did not perform an analysis of circulating EPC, we were also consistent with MMP-9 playing a role in the recruitment of bone marrow–derived inflammatory cells into the tumor. The presence of CD31/PECAM and EGFP coexpressing cells in the tumor microvasculature of mice transplanted with MMP-9+/− bone marrow (groups 1 and 2) was also consistent with MMP-9 playing a role in the recruitment of bone marrow–derived endothelial cells into the tumor stroma. Because we did not perform an analysis of circulating EPC, we cannot conclude at this point whether MMP-9 is also involved in the release of EPC from the bone marrow into the circulation in our model. However, the data show that the presence of bone marrow–derived endothelial cells in the tumor vasculature depends on MMP-9 expression.

MMP-9 has been shown to contribute to tumor angiogenesis in a complex way. Most of the mechanisms have pointed to endothelial cells where MMP-9 promote their ability to form tubes on Matrigel (31), trigger an angiogenic switch by increasing the solubilization of VEGF (13), or as discussed above, by promoting the mobilization and recruitment of EPC. Using the solubilization of VEGF (13), or as discussed above, by tubes on Matrigel (31), trigger an angiogenic switch by increasing endothelial cells where MMP-9 promote their ability to form marrow–derived endothelial cells in the tumor vasculature. Upon stimulation by VEGF, endothelial cells express platelet-derived growth factor (PDGF) B, which stimulates the migration and proliferation of PDGF receptor β expressing pericytes (33). Pericytes also express epithelial growth factor (EGF) receptor ErbB1 and ErbB2 and endothelial cell express heparin-bound EGF upon exposure to angiopoietin-1 (34). It is conceivable that MMP-9 promotes pericyte recruitment along endothelial cells by increasing the solubility of PDGFB or heparin-bound EGF, a possibility currently explored in our laboratory.

Pericytes are present in most tumor blood vessels but are often abnormal (35). Interestingly, they have been recently identified as a novel and important target for antiangiogenic therapies. Bergers et al. have shown in a mouse model of pancreatic islet cancer that targeting only VEGFR in endothelial cell with the receptor tyrosine kinase inhibitor SU5416 is effective against early-stage tumors but not against large and well-vascularized tumors. In contrast, a PDGF receptor kinase inhibitor SU6668 targeting pericytes is effective in end-stage tumors and a combination of VEGFR and PDGFR inhibitors has the maximum antiangiogenic effect (36). A similar observation has been made in a rat brain tumor model (37). Targeting both endothelial cells and pericytes or blocking pericyte recruitment to the tumor vessels may therefore be a powerful antiangiogenic strategy by inducing endothelial cell apoptosis and tumor vessel regression. This approach could be particularly effective in tumors that exhibit mature vessels rich in pericytes. It is conceivable that inhibiting pericytes in the tumor vasculature might negatively contribute to tumor progression by increasing vascular permeability and facilitating the diffusion of cytotoxic drugs.

It is interesting to note that our analysis of human neuroblastoma tumors points to a deficit in angiogenesis in favorable histology tumors that are also rich in Schwannian stroma. Other investigators have shown that Schwann cells actively contribute to inhibition of angiogenesis by secreting antiangiogenic factors like tissue inhibitor of metalloproteinase inhibitor-2, pigment epithelium-derived factor, and SPARC (38).

The same group of investigators recently reported that treatment of mice with a folded synthetic peptide corresponding to the epidermal growth factor-like module of SPARC decreases the size and the density of microvessels that formed in a plug of Matrigel implanted s.c. (39). Consistent with their data, we also observed smaller blood vessels in favorable histology tumors which contained Schwann cells when compared with unfavorable histology tumors which did not contain Schwann cells and had the same endothelial cell area but contained larger and more mature vessels at a lower density. The observation that a more aggressive type of tumor (unfavorable histology) has a lower microvessel density than a less aggressive type illustrates how the measurement of microvessel density along cannot be considered as a single and reliable marker for angiogenesis. As pointed out by several laboratories, microvessel density alone is not a measure of the angiogenic dependence of a tumor and a high vascular density does not necessarily imply a rapid tumor growth (40). The degree of maturation of blood vessels as examined by their degree of coverage by pericytes may thus

![Figure 7](image-url)

Figure 7. Primary neuroblastoma tumors with an unfavorable histology have higher infiltration with MMP-9 expressing inflammatory cells. Representative pictures of primary human neuroblastoma tumors immunostained for human MMP-9 (red) and SMA (green) in (A) and for MMP-9 (red) and Lysozyme (green) in (B). Bar, 50 μm. Inset, high magnification of coexpression of MMP-9 and lysozyme. C, average number of MMP-9 expressing cells per field from 20 microscopic fields examined in each tumor.
provide an additional indicator of tumor aggressiveness and suggest a potential benefit from antiangiogenic therapies that target pericytes.

The significance of our observation in the context of clinical treatment of neuroblastoma should be discussed. Many patients with high-risk neuroblastoma are treated with intensive myeloablative chemotherapy followed by bone marrow transplantation (41). Intensive myeloablative chemotherapy is typically followed by an increased mobilization of bone marrow cells and EPC into the peripheral blood circulation (42). As shown by our data, these cells contribute to tumor progression by promoting the formation of a more mature vascular. By demonstrating that MMP-9 is critical for these cells into the tumor stroma, our data suggest that inhibition of MMP-9 activity after bone marrow transplantation may be of therapeutic benefit by preventing the infiltration of residual tumors by bone marrow–derived cells. Inhibition of MMP-9 may also interfere with the recruitment of pericytes along newly formed microvessels.

In summary, our data provide a new insight into the contribution of bone marrow–derived cells to tumor angiogenesis in which MMP-9 plays a key role.

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