Chapter 51
Bone Marrow-Derived Flk-1–Expressing CD34+ Cells Contribute To the Endothelium of Tumor Vessels in Mouse Brain

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INTRODUCTION

Angiogenesis is required for sustained neoplastic growth (3). The sprouting of new vessels from the pre-existing vasculature supplies a proliferating mass of hypoxic tumor cells with oxygen and nutrients. Glioblastoma multiforme is one such tumor, characterized by extensive vascular growth and remodeling. A permissive microenvironment coupled with tissue hypoxia, which promotes elaboration of the angiopoietins and vascular endothelial growth factor (VEGF), is implicated in tumor neovascularization. Angiopoietins seem to stimulate recruitment of bone marrow¡Vderived endothelial precursor cells, and VEGF induces myeloproliferation and increases circulating levels of endothelial progenitor cells (2) and also contributes to neo-angiogenesis. The receptor to which VEGF binds, Flk-1 (the mouse variant of the human KDR), is expressed on endothelial cells and helps trigger vascular proliferation when activated.

Hemangioblasts, the precursors of both the hematopoietic and the endothelial cell lineages, are recruited from stem cell reservoirs within the bone marrow and may play a significant part in tumor angiogenesis (8, 9). These hemangioblasts carry the phenotypic markers of hematopoietic stem cells and differentiation-stage hematopoietic/endothelial cells (CD34) (4, 13). Once in peripheral blood, hemangioblasts migrate to sites of tissue ischemia, trauma, or tumor growth, where they can differentiate into endothelial cells to support vascular remodeling (1, 5, 7, 11, 12, 14).

In this chapter, we report identifying and isolating a subpopulation of bone marrow¡Vderived cells thought to be responsible, in part, for vessel restructuring in the presence of brain tumors. These cells are positive for CD34 and Flk-1, and are morphologically indistinguishable from endothelial cells of the neovasculature, with which they are closely associated. Our results suggest that bone marrow cells are recruited to newly formed and remodeled tumor vessels. Their recruitment may occur in response to signals from a highly proliferating milieu and their role is to support the neovascular complex and to promote tumor growth.

MATERIALS AND METHODS

Animal Model and Surgery

A subclone of the rat glioblastoma line RT-2 (RAG/RT-2) was used in this study. This cell line was adapted to grow in T-cell¡Vdepleted RAG2/KO-5.2 mice. T-cell¡Vdepleted mice of the RAG-2/KO5.2 line were lethally irradiated (see Fig. 51.1). Their bone marrow was rescued with marrow cells from GFP+ transgenic C57BLKa-Thy-1.1 mice. These animals were allowed to recuperate for 3 to 4 weeks before tumor implantation. Ten thousand RAG/RT-2 cells in 10 ƒÝl of Dulbecco¡¦s modified Eagle¡¦s medium (DMEM) were stereotactically injected into the left striatum (2 mm lateral to and 1 mm posterior to Bregma) of the chimeric mice using a mouse stereotactic frame. The resultant tumors had growth patterns and histology similar to that induced by RT-2 cells in rat brains. The animals were killed on Days

3, 6, 9, or 12 after surgery. After being perfused with cold saline buffer followed by a 4% neutral paraformaldehyde phosphate buffer (PFPB) at 4¢XC, their brains were cut into 40-ƒÝm serial sections on a freezing microtome. A total of four animals were used for each time point. Control animals were injected with DMEM only.

Immunofluorescence and Confocal Microscopy

Brain sections were blocked with normal donkey serum and were incubated with a combination of primary antibodies: anti-mouse CD34, anti-human von Willebrand factor, anti-mouse CD45, and anti-mouse Flk-1 (TO14, a gift from Dr. Rolf A. Brekken). This was followed by incubation with species-specific secondary antibodies conjugated with Cy3, Cy5, or Texas Red. Sections were mounted on slides and counterstained with Vectashield mounting medium containing 4¡¬,6-diamidino-2-phenylindole (DAPI) for nuclear detection. Slides were viewed with an Axioplan2 Zeiss fluorescence microscope and a Zeiss LSM510 two-photon laser scanning confocal microscope. The region of tumor growth was identified by a dense DAPI nuclear staining pattern. Endothelial cells were detected by antibody combinations: anti¡VFlk-1 and anti-CD34 (for progenitor cells), and anti-CD45 (to determine hematopoietic origin). Cells in six random fields (150 ¡Ñ 150 ƒÝm2) were counted per section, as detailed elsewhere (15). Sections were scanned at 1-ƒÝm intervals and step z-series were obtained using the accompanying image analysis software (Axiovision). The quantification of the cells and three-dimensional (3-D) reconstruction of the vessels was done with Volocity software (Improvision).

GFP+/CD34+/Flk-1+ cells were counted as a percentage of total endothelial cells in blood vessels within the tumor core, to gauge the extent to which bone marrow¡Vderived cells were recruited to the vasculature. Statistical analysis by analysis of variance (ANOVA) and Student¡¦s t test were used to ascertain whether a significant proportion of GFP+ endothelial cells in fact expressed Flk-1 and CD34 (95% confidence interval). A total of 506 sections were examined in this study. For the GFP+/CD34+/Flk-1+ cells in question to be considered as putative endothelial-like cells, they must have been situated along the intimal vascular wall and elongated in morphology.

Fluorescence-Activated Cell Sorter (FACS) Analysis

The cell sorting/analysis protocol was essentially the same as that used to harvest hematopoietic stem cells. In brief, at Day 12 after tumor engraftment, the animals were anesthetized and perfused transcardially with 25 ml of cold (4„aC) saline to flush out intraluminal blood. The brain was harvested under sterile conditions and placed into dissection medium (Hank¡¦s buffered saline solution, HBSS, with 0.5 mmol/L ethylenediamine tetra-acetic acid, EDTA, with 1% penicillin and streptomycin). The tumor, along with a 1-mm peritumoral margin, was microdissected out on a cold stage. The tumor tissue was minced and incubated in HBSS together with 0.1% collagenase, 0.1% hyaluronidase, 2500 U/ml of DNase, and 3 mmol/L of CaCl2 for 1 hour. Cells were filtered through 70-ƒÝm and 40-ƒÝm filters, spun down, and resuspended in HBSS. Aliquots of 5 million cells were labeled with biotinylated anti-CD34 and anti¡VFlk-1 primary antibodies. These cells were washed in HBSS at 4„aC and were secondarily labeled with Texas Red-conjugated streptavidin (for CD34) and PE-conjugated, species-specific anti¡VFlk-1 antibody. Before FACS analysis, propidium iodide was added to remove dead cells during cell sorting by gating. Cells were fractionated by a FACSVantage SE cell sorter machine, with calibration and compensation for the fluorochromes using the appropriate isotype controls. Cell sorting was based on 95% fluorescence thresholds.

RESULTS

We identified a population of bone marrow¡Vderived cells that express both CD34 and Flk-1 on their surface. These cells localize to the region of the growing tumor. Tumor size expanded from 0.18 ¡Ó 0.12 mm3 at Day 3 to 0.97 ¡Ó 0.67 mm3 at Day 6; 3.55 ¡Ó 1.04 mm3 at Day 9; and 16.60 ¡Ó 2.48 mm3 at Day 12 (see Fig. 51.1B). By Day 12, tumor invasion had become quite diffuse, though confined to the injected hemisphere.

The vascular supply to the growing tumor region also developed extensively during much of the time course. Whereas, at Day 3, few vessels were observed in the tumor (18.2 ¡Ó 6.5 vessels/mm2); by Day 6, a sharp increase in vessel number was apparent (47.9 + 8.3 vessels/mm2). At Day 9, there were, on average, 49.2 + 9.9 vessels/mm2. There was a slight but significant reduction in vascular density at Day 12 (31.6 + 7.3 /mm2; see Fig. 51.1C). There was an increase in the caliber of tumor vessels from Day 3 through Days 6 to 12. At Day 3, vessel caliber averaged 11.0 ¡Ó 2.8 ƒÝm, and increased to 15.0 ¡Ó 4.1 ƒÝm at Day 6, where it leveled off (Day 9, 15.2 ¡Ó 3.9 ƒÝm; Day 12, 15.5 ¡Ó 4.5 ƒÝm). Vessels of smaller caliber were more commonly observed at the periphery of the tumor rather than the core of the tumor. The scatter was most pronounced at Day 9. The scatter coincided with the onset of the expansion of the tumor mass, and was preceded by the increase in vascular density. It was noteworthy that, by Day 12, the correspondence between increase in tumor size and concomitant increases in vessel density and caliber was no longer apparent. At the later stage of tumor growth, the vessel density declined despite continued tumor expansion and increased average vessel diameter. Histologically, the tumors showed substantial necrosis and hemorrhage, which is attributable to possible pruning and involution of the vessels that had arisen earlier.

The number of GFP+ (i.e., bone marrow¡Vderived) CD34-expressing cells within the vascular tree of the tumor increased over time. At Day 3, minimal GFP immunoreactivity was evident in the region of brain tumor¡Xonly 10% of tumor vessels contained at least one GFP+ cell of endothelial morphology. By Day 6, GFP+ endothelial-like cells were found in 35% of tumor vessels. At Day 9, the percentage had increased to 46%; and by Day 12, 54% of tumor vessels contained one or more GFP+ cells of endothelial morphology. On the contralateral hemisphere not containing tumor, however, the number of GFP+ and GFP+/CD34+ cells was minimal, and in the control brain, only four GFP+ cells were counted in the entire striatum, and less than 2% of the vessels were populated by GFP+/CD34+ cells. Despite the fact that 54% of its vessels were eventually found to harbor at least one GFP+/CD34+ cell in their endothelial lining, only a small fraction of the GFP+/CD34+ cells in newly formed blood vessels within the tumor region were also immunoreactive for Flk-1, although the proportion of total endothelial cells immunoreactive for GFP, CD34, and Flk-1 increased consistently during the time course. At Day 3, less than 1% (0.8 ¡Ó 0.3%) of the cells in tumor vessels was immunoreactive for all three markers. At Day 6, approximately 1.5% (¡Ó 0.9%) of cells in tumor vessels were triple immunoreactive. By Day 9, the percentage of triple-immunoreactive cells had climbed to 3.7% (¡Ó 1.2%); this increased to 4.2% (¡Ó 1.2%) by Day 12 (see Fig. 51.2A). These cells were incorporated into the vasculature, as confirmed by optical dissection using confocal microscopy. Some of the cells localized to the branching points of the vessels (see Figs. 58.2 and 58.3).

It was noteworthy that a small number of triple-immunoreactive cells were more round in shape. In this study, cells with this morphological feature were excluded from the count of endothelial-like cells. However, we acknowledge that there may exist a continuum of morphological changes between circulating cells lately arrested on the luminal wall and those that had been incorporated into the vascular tree. GFP+/CD34+/Flk-1+ cells were never found outside of the vascular wall. Likewise, no evidence could be found of a predilection of these cells for vessels of a specific

caliber.

Furthermore, all cells that were GFP+ and of endothelial-like appearance were noted to express von Willbrand factor (vWF) and the pan-hematopoietic marker, CD45. Additionally, a multitude of extravascular GFP+ cells in proximity to tumor vessels were identified as CD45 immunoreactive. A majority of these cells resembled macrophages and monocytic cells. We presume that these cells had been recruited into the tumor stroma. Their role in tumor progression and angiogenesis has yet to be elucidated.

Collectively, our results show that a small population of bone marrow¡Vderived CD34+/Flk-1+ cells was recruited into the vascular wall. They accounted for a minor percentage of GFP+/CD34+ cells isolated from the tumor mass. Nevertheless, they represent a distinct population of cells, verified by flow cytometry, and effectively play a role in the development of the tumor neovascular tree.

DISCUSSION

Our findings suggest that the growing brain tumor incorporates bone marrow¡Vderived cells into its neovasculature. Our mouse model permitted visualization of GFP+ cells from bone marrow after irradiation and marrow transplant. We examined the nature of these GFP+ cells in the brain tumor region both at the molecular and morphological level and identified a population of cells within the bone marrow with a tropism for tumor neovasculature that carried the endothelial phenotype.

GFP+ cells, in general, were found to preferentially migrate to the tumor region. The majority of GFP+ cells in the tumor were in close proximity to the vessels but were not incorporated into the vascular wall. The true identity of these cells remains unclear. Likely, they are involved in the host inflammatory response to the expanding tumor. Only a small percentage of GFP+ cells assumed an endothelial morphology, carried the CD34 surface marker, and co-expressed Flk-1 (0.5 ¡Ó 0.4%). Vajkoczy et al. reported that extravasation of circulating marrow-derived cells is an important first step in the sprouting of new vessels within and around the tumor (16, 17). It is plausible that, in this tumor model, the population of GFP+ but CD34 and Flk-1 negative cells in the extravascular compartment consists of a combination of cells that correspond to the leukocytes and auxiliary vascular cells described by Vajkoczy. Circulating endothelial progenitor cells are said to become arrested in the tumor vasculature, whereupon they burrow through the vessel wall to colonize and divide in the interstitium in clusters before building new vascular sprouts or incorporating into nearby vessels.

Tumor-derived cytokines, such as VEGF and transforming growth factor (TGF)-ƒÒ, have been shown to induce myelopoietic stimulation and mobilization of CD34+ progenitor cells. These CD34-expressing cells incorporate as endothelial cells into newly formed vessels in response to ischemia. In our study, CD34-expressing cells were found almost exclusively within the growing tumor, suggesting that the development of new vessels was most pronounced here, as anticipated. As the tumor grew, the percentage of its CD34+ vessels that contained at least one GFP+ marrow-derived cell in the endothelium also increased, to more than 50% by Day 12. This accounted for 4% of all endothelial cells within the walls of these vessels. Eventually, bridging of endothelial cells within engorged tumor capillaries and venules is thought to result in multiple small vessels from one source (6). This phenomenon may explain the presence of triple-immunoreactive endothelial cells over a broad range of vessel diameters in later days.

Furthermore, when GFP+ cells from brain tumors were analyzed for the expression of endothelial markers using flow cytometry, there was a subpopulation of cells that were intensely immunoreactive for Flk-1 and CD34. Emerging evidence suggests that endothelial progenitor cells, such as the angioblasts, share a number of antigenic determinants with hematopoietic progenitor cells, including CD34, Flk-1, and Tie-2. The angioblasts are the precipitant for physiological and pathological vasculogenesis. Theoretically, angioblasts should not express CD45. The presence of the pan-hematopoietic marker, CD45, on the endothelial-like cells that we identified suggests that these cells are of hematopoietic origin. Additionally, it has been shown that myeloid progenitor cells are present within tumor tissue and are strongly immunoreactive for CD34, in contrast to the faintly immunoreactive endothelial cells. We suspect that our population of bone marrow¡Vderived GFP+/CD34+/Flk-1+ cells is of myeloid lineage (18).

Although other groups have recently downplayed the contribution of bone marrow¡Vderived cells in reconstituting the tumor or ischemic neovasculature, we think that the function of these cells is to enrich the vascular niche of the growing tumor and to augment tumor expansion (10, 19).

The potential clinical implications of this research abound. Glioblastoma multiforme is a lethal disease. Surgical, chemotherapeutic, and radiotherapeutic intervention have, thus far, proved inadequate. Our findings in the laboratory suggest that a future goal of treatment might involve use of the bone marrow¡Vderived cells identified in our model as a means of targeting the tumor region with growth-inhibiting agents. These CD34+/Flk-1+ cells may be extracted from the marrow, genetically manipulated to express cytotoxic factors, and introduced into the peripheral circulation. Although ultimately constituting a mere 4% of the tumor vascular endothelial cell population, these marrow-derived cells seem to serve a permissive function in allowing sprouting of new vessels. The inhibition of neovascularization because of altered targeting of marrow-derived cells to the tumor or because of elaboration of cytotoxic factors by these marrow-derived cells might prevent the growth and spread of the nascent tumor, and, thereby, make neurosurgical excision a more successful therapy.

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FIG. 51.1 *A*, schematic depiction of the murine model used in this study. GFP+ cells from C57BLKa-Thy-1.1 mice were used to repopulate the marrow of lethally irradiated animals of the RAG-2/KO5.2 line. RAG/RT-2 glioma cells were injected stereotactically into the left striatum of mice that had undergone marrow transplantation. *B*, tumor size is shown to increase consistently during the experimental time course. At Day 3 after tumor implantation, the tumor occupied a volume of 0.18 ± 0.12 mm^3. This volume increased to 16.6 ± 2.48 mm^3 at Day 12. *C*, the increase in tumor volume was preceded by an increase in the vascular density within the tumor mass. There was a decline in vascular density at Day 12, which was attributed to the involution of vessels and an increase in tumor necrosis.

FIG. 51.2 Contribution of bone marrow–derived cells to the tumor vascular endothelium. *A*, GFP+/CD34+/Flk-1+ endothelial-like cells incorporate into the vascular tree of the tumor region. During the experimental time course, an increase was noted in the contribution of marrow-derived cells to the tumor vascular endothelium. By Day 12, approximately 4% of all endothelial cells in the tumor region were GFP+/CD34+/Flk-1+. *B*, *C*, and *D*, montage taken from confocal microscopy depicts tumor vessels in cross section, taken from different points of tumor growth. There was an increase in GFP+/CD34+/FLK-1+ cellularity in the tumor vasculature with time. Confocal micrographs, taken at a 1-mm optical thickness: *B*, 3 days after surgery; *C*, 9 days after surgery; *D*, 12 days after surgery. GFP+ bone marrow–derived cells appear green. DAPI was used to counterstain the nuclei (blue). These montages show immunolabeling with CD34 (red) and Flk-1 (yellow) in the same vessel. *Arrows* point to endothelial cells. Scale bar: 30 mm in *B*; 50 mm in *C* and *D*.

FIG. 51.3 GFP+/CD34+/FLK-1+ endothelial-like cells are most evident at Day 9 after tumor implantation. In this montage, all six panels refer to the same field of view, through different filters. Three GFP+/CD34+/FLK-1+ endothelial-like cells are indicated by arrows. The lowest arrow refers to a blind loop, where a new vessel is in the process of sprouting. In the panel farthest to the right, *z*-stack analysis of the endothelial cell in question confirms its localization to the endothelial lining οφ τηε βλοοδ ϖεσσελ. Σχαλε βαρ: 50 μm.