Vasculogenesis Plays a Role in the Growth of Ewing's Sarcoma *in Vivo*[1]

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ABSTRACT

Vasculogenesis, the process by which endothelial cell precursors are recruited and organized to form a vasculature, has traditionally been thought to play a role only in embryonic development. However, several studies have now been published suggesting that vasculogenesis may have a role in the formation of new vascular networks during postnatal life. Recent studies suggest the existence of circulating endothelial precursor cells that arise from outside the place of vascularization. Using a mouse bone marrow (BM) transplantation model that takes advantage of MHC haplotype differences between donor and recipient mice, we examined the contribution of donor BM-derived cells to neovascularization in recipient nude mice with developing Ewing's sarcoma tumors. We found that the donor BM cells gave rise to endothelial cells *in vitro* and colocalized with neovessels in Ewing's sarcomas *in vivo*. We also found that donor BM-derived cells were involved in the formation of the tumor vasculature. Our findings indicate that not only angiogenesis but also vasculogenesis was involved in the development of Ewing's sarcoma in our mouse model.

INTRODUCTION

Vasculogenesis is the process by which endothelial cell precursors derived from mesodermal cells are recruited and organized to form a primitive vessel network (1, 2). This process has typically been described during embryogenesis, when extraembryonic mesodermal cells differentiate to form blood islands composed of angioblasts at the periphery and primitive hematopoietic cells at the center (3).

Angiogenesis is the process by which new blood vessels are formed from preexisting vessels. This process has been thought to be primarily responsible for the generation of new vessels during postnatal life in response to pathological conditions such as tumor formation, rheumatoid arthritis, and retinopathies and in physiological processes such as normal hair growth and the formation of the corpus luteum during the estral cycle (4–6).

Several studies have now been published that suggest that not only angiogenesis but also vasculogenesis may be involved during postnatal life in situations that require an expanded vessel network. Studies in the 1960s demonstrated the presence of circulating endothelial cells in Dacron grafts inserted into arteries of pigs, rabbits, and dogs (7, 8). In 1971, investigators reported spindle-shaped cells in the arterial intimal thickening in an allografted human heart. These cells were of both donor and host origin, with the host cells originating from circulating precursors (9). In 1993, endothelial cells were identified on the lining of a ventricular assist device (10). Putative progenitor endothelial cells have recently been isolated from peripheral blood and shown to be incorporated into sites of neovascularization in ischemia models (11). Finally, in 1998, the endothelialization of vascular prostheses by marrow-derived cells was reported (12). All of these studies point toward the existence of circulating endothelial precursor cells that arise from outside the place of vascularization. Furthermore, these data suggest that both angiogenesis and vasculogenesis may contribute to the formation of new vasculature during adult life.

Ewing's sarcoma is a primitive neuroectodermal tumor that most often affects children and young adults between 5 and 30 years of age (13). Despite multiple attempts to improve the efficacy of chemotherapy for this disease, the 2-year metastasis-free survival rate for patients with Ewing's sarcoma has not improved over the past 15 years (14–18). New therapeutic approaches are therefore needed to reduce the mortality rate. Understanding of the biology and mechanisms involved in Ewing's sarcoma tumor growth and progression may lead to the identification of new therapeutic targets.

We demonstrate here that not only local endothelial cells but also BM[3]-derived cells are involved in the generation of the new tumor vasculature during the growth of Ewing's sarcoma. Our findings indicate that not only angiogenesis but also vasculogenesis is involved in the development of new vasculature in Ewing's sarcoma.

MATERIALS AND METHODS

Ewing's Sarcoma Cells. TC71 human Ewing's sarcoma cells, kindly provided by Dr. P. Pepe (University of Southern

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[3] The abbreviations used are: BM, bone marrow; BMT, bone marrow transplantation; IL, interleukin; DAB, 3,3′-diaminobenzide hydrochloride; AcLDL, acetylated low-density lipoprotein; VEGF, vascular endothelial growth factor.

California, Los Angeles, CA), were cultured in Eagle's modified essential medium with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM nonessential amino acids, 1 mM penicillin-streptomycin, and 2-fold vitamin solution (Life Technologies, Inc., Grand Island, NY). Cells present the Ewing's sarcoma t(11;22) as detected by reverse transcription-PCR (19). Cells were free of *Mycoplasma* as verified with the use of Gen-Probe screening (Gen-Probe, San Diego, CA).

Mice and Murine Bone Marrow Cells. Male nude BALB/cAnN mice, which have the MHC strain-specific surface antigen d (H-2^d), and C57BLxBALB/c F1 (CB6 F1) mice (H-$2^{b/d}$), 8–13 weeks of age, were purchased from the National Cancer Institute and housed in a specific pathogen-free facility. CB6 F1 mice were used as BM donors, and BALB/cAnN mice were used as BM recipients.

Bone marrow cells were isolated from donors by flushing the hind femur, and cell samples were plated for 24 h to deplete them of adherent, fully differentiated endothelial cells. The nonadherent cells were plated on fibronectin (Sigma, St. Louis, MO), and their morphological evolution was examined daily during the time of culture (2 weeks). Cells were cultured in endothelial basal medium supplemented with human epidermal growth factor, bovine brain extract, and 5% fetal bovine serum with or without hydrocortisone (Clonetics-Biowhittaker, Walkersville, MD). After 2 weeks, cultures were analyzed for the expression of CD105 (endoglin), an endothelial marker, and CD80, a marker of monocyte and dendrite lineage. For E-selectin detection, tumor necrosis factor α (R&D Systems, Minneapolis, MN) was added to the cultures for 4 h, after which time the cultures were analyzed for E-selectin expression. Cultured cells were analyzed for their capacity to uptake fluorescent AcLDL (DiI-AcLDL; Biomedical Technologies Inc., Stoughton, MA) following the manufacturer's instructions.

Staining of Donor Bone Marrow Cells. Freshly isolated BM cells were incubated with the fluorescent cell tracker dye CM-DiI (Molecular Probes, Eugene, OR) at a concentration of 10 mM for 5 min at 37°C and then for 15 min at 4°C. Cells were washed and resuspended in fresh HBSS immediately before use. Cells were kept on ice and protected from light until they were injected into the tail veins of BALB/c tumor-bearing mice, within 1 h after staining. TC71 human Ewing's sarcoma cells and LM6 human osteosarcoma cells were used as non-BM control cells. Cells were stained with the fluorescent cell tracker dye CM-DiI as described above and injected into the tail veins of BALB/c tumor-bearing mice within 1 h after staining.

BMT. Recipient mice were irradiated using an external cesium source (^{137}Cs Mark 1 irradiator; J. L. Shepherd & Associates, Glendale, CA). Mice underwent whole-body irradiation with 9.5 Gy in two doses given at least 3 h apart. Freshly isolated BM cells (10^6/200 μl) from CB6 F1 mice were injected via the lateral tail vein into nude BALB/cAnN recipients within 4 h after irradiation. From the day before BMT until 15 days after transplantation, recipient mice received acid water with neomycin (2 g/liter; Sigma). Reconstitution of recipients was assessed with surface staining for strain-specific H-2 surface antigens specific to the donor MHC (presence of donor H-$2k^b$- and I-A^b-positive staining) and by PCR analysis of the presence of donor IL-1β gene in spleens and mononuclear cells isolated from peripheral blood (see "PCR Analysis of IL-β" in "Materials and Methods").

Ewing's Sarcoma Tumor Model. Nude BALB/cAnN (H-2^d) recipient mice that had received BM transplants from CB6 F1 (H-$2^{b/d}$) mice were injected s.c. with TC71 Ewing's sarcoma cells plus Matrigel (10^6 cells/in 0.5 ml of Matrigel), TC71 cells alone (2.5×10^6 cells in 0.2 ml of HBSS), or 0.5 ml of Matrigel alone 4 weeks (32 days) after BMT. Tumors of about 3–4 mm in diameter can be detected 6 days after injection of TC71 cells.

Immunohistochemical Analysis. Mice were sacrificed 5 weeks (38 days) after BMT. Samples of spleen and s.c. Ewing's sarcoma tumors were placed in Optimal Cutting Temperature compound (Sakura Finitek USA Inc., Torrance, CA), frozen in liquid nitrogen, and stored at −80°C. For the detection of class I MHC of donor origin, cryostat sections were fixed in acetone, blocked for endogenous peroxidase with H_2O_2, and incubated successively with 4% gelatin in PBS, avidin-biotin blocking kit (Vector, Burlingame, CA), and biotinylated mouse antimouse H-$2K^b$ antibody (PharMingen, San Diego, CA). After avidin-peroxidase incubation, sections were developed with DAB.

For the detection of class II MHC of donor origin, cryostat sections were fixed in acetone, blocked successively with H_2O_2 and Fab fragments, and incubated with normal horse serum and normal goat serum. Sections were subsequently incubated with mouse antimouse I-A^b antibody (PharMingen) and with rat antimouse IgG2a horseradish peroxidase-conjugated antibody (Serotec, Raleigh, NC). Sections were developed with DAB.

For the double detection of CD31/platelet endothelial cell adhesion molecule and H-$2K^b$, sections were fixed in acetone, blocked with normal horse serum and normal goat serum, and incubated with CD31 rat antimouse antibody (PharMingen) and goat antirat Texas Red antibody (Jackson, West Grove, PA); samples were successively blocked with 4% gelatin in PBS and avidin-biotin blocking kit (Vector) and incubated with biotinylated mouse antimouse H-$2K^b$ antibody and streptavidin-FITC (PharMingen).

For colocalization of Meca-32 in tumors excised from mice previously injected with CM-DiI-stained BM cells, frozen sections were fixed in 1% paraformaldehyde, blocked for endogenous peroxidase with H_2O_2, and incubated successively with normal horse and goat serum, rat antimouse Meca-32 (PharMingen), and goat antirat horseradish peroxidase-conjugated antibody (Jackson). Sections were developed with DAB.

For the detection of CD105 (endoglin), cultured cells were fixed in acetone, blocked with H_2O_2, and incubated with normal horse serum and normal goat serum. Sections were then incubated with rat antimouse CD105 antibody (PharMingen) and subsequently incubated with goat antirat antibody conjugated with horseradish peroxidase (Jackson). Sections were developed with DAB.

For E-selectin detection on cultured cells, cultures were fixed in acetone, blocked with normal horse serum and normal goat serum, and incubated with rat antimouse E-selectin antibody (PharMingen). Subsequent detection was performed with goat antirat Texas Red (Jackson).

Quantitation of Bone Marrow-derived Vessels. Each stained section was screened at ×10 (eyepiece) and ×4 (objective) magnification. Vessels were counted at ×20 (objective)

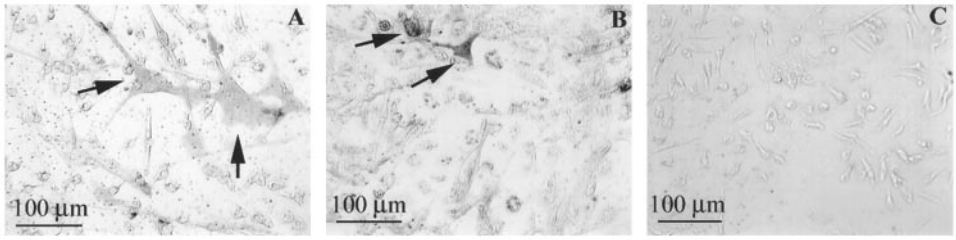

Fig. 1 Endoglin (CD105) staining in *in vitro* cultures of BM-derived cells. Whole BM cell samples were plated for 24 h to deplete them of adherent, fully differentiated endothelial cells. Nonadherent cells were plated on fibronectin. After 2 weeks, cultures were stained for endoglin (CD105). *A*, culture without hydrocortisone; *B*, culture with hydrocortisone; *C*, negative control. *Arrows* indicate CD105-positive cells.

magnification. All vessels were counted on each sample. Branching structures were considered as a single vessel. The percentage of BM-derived microvessels for each sample was expressed as the number of BM-derived vessels (green staining) divided by the number of total vessels (red staining) multiplied by 100.

Preparation of Genomic DNA. Samples of spleen from chimeric mice were snap-frozen in liquid nitrogen and stored at −80°C until DNA isolation. Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. DNA was extracted from spleen and peripheral blood from CB6 F1 (donor) and BALB/c (recipient) mice and from BALB/c chimeras that had received transplants of BM from CB6 F1 mice. Peripheral blood was subjected to a density gradient with Hystopaque-1083 (Sigma) to obtain a mononuclear cell layer according to standard methods. DNA was extracted from the mononuclear cell layer using the QIAamp DNA Mini Kit. Genomic DNA from livers of BALB/c mice was purchased from Clontech (Palo Alto, CA).

PCR Analysis of IL-1β. Engraftment of donor BM was verified by PCR analysis of a polymorphic microsatellite region within the murine IL-1β gene. The primer sequences were 5′-CCAAGCTTCCTTGTGCAAGTA-3′ and 5′-AAGCCCAA-AGTCCATCAGTGG-3′. The oligonucleotides were synthesized by Genosys (Sigma). Amplification was performed with an initial step of 1 min and 30 s of denaturation at 95°C; 35 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 57°C, and 1 min of elongation at 72°C; and one last cycle of 30 s of annealing at 57°C and 7 min of elongation at 72°C. Conditions for the PCR were 50-μl total volume with 200 ng of genomic DNA as template, 0.1 μM primers, 0.4 mM deoxynucleotide triphosphate, 1× PCR buffer/Mg^{2+}, and 2.5 units of Taq DNA polymerase (Roche, Nutley, NJ).

RESULTS

Analysis of Bone Marrow Cells *in Vitro*. We tested the ability of BM cells to give rise to endothelial cells *in vitro*. Bone marrow cells were flushed from donor mouse femurs, and then the cell samples were plated for 24 h to deplete them of adherent, fully differentiated endothelial cells. The nonadherent cells were plated on fibronectin, with or without hydrocortisone, a hormone that stimulates endothelial cell differentiation. After 2 weeks, the cultures were analyzed for CD105 (endoglin) and CD80 staining. The cells cultured with hydrocortisone presented stronger positive staining for CD105 (endoglin) than did the cells cultured without hydrocortisone (Fig. 1). The cells cultured without hydrocortisone were positive for CD80, a marker of monocyte and dendrite lineage, whereas the cells cultured with hydrocortisone were negative for CD80 (data not shown). This indicates that the culture conditions with hydrocortisone potentiated a stronger expression of CD105 on the cells present in the culture. Fluorescence-activated cell-sorting analysis and immunohistochemical staining showed that the cells obtained after plating the whole BM (nonadherent cells) lacked CD105 (data not shown), which indicated that this phenotype appears later after the given culture conditions. The cells present in the hydrocortisone cultures had the ability to uptake AcLDL, a characteristic associated with an endothelial phenotype. When tumor necrosis factor α was added to the cultures, E-selectin was overexpressed in the cultured cells. This is a specific characteristic of endothelial cells (data not shown). These data demonstrate that mouse BM cells have the ability to give rise to endothelial-like cells, given the appropriate microenvironment conditions (in this case, represented by the culture conditions).

Localization of Donor Bone Marrow Cells in the Neovasculature of Newly Formed Ewing's Sarcomas. Whole BM cells were stained with CM-DiI to permit tracking of these cells. CM-DiI-stained whole BM cells were injected in the tail veins of syngeneic BALB/c mice that had been inoculated s.c. with TC71 Ewing's sarcoma cells 6 days earlier (Fig. 2*A*).

Frozen sections of tumors removed 6 days after BM cell injection were stained with Meca-32, a pan-endothelial marker (20). Bright-field microscopy and fluorescence microscopy were then used to detect Meca-32-stained microvessels and CM-DiI-positive cells, respectively. Meca-32-stained microvessels and fluorescent donor-derived (CM-DiI-positive) BM cells were found to colocalize (Fig. 2*B*). Labeled BM cells were located close to and within the microvessels of the tumor. Together, these data suggest that circulating BM cells are attracted to the sites of neovascularization in the rapidly growing Ewing's sarcoma.

Vasculogenesis in Ewing's Sarcoma. We next used a BMT model to determine the contribution of donor BM cells to vascular development over a longer period of time. We exploited MHC haplotype differences between donor mice and recipient mice to distinguish cells of donor origin from cells of the host. Engraftment of donor BM cells in recipient mice was assessed by immunohistochemical analysis of strain-specific

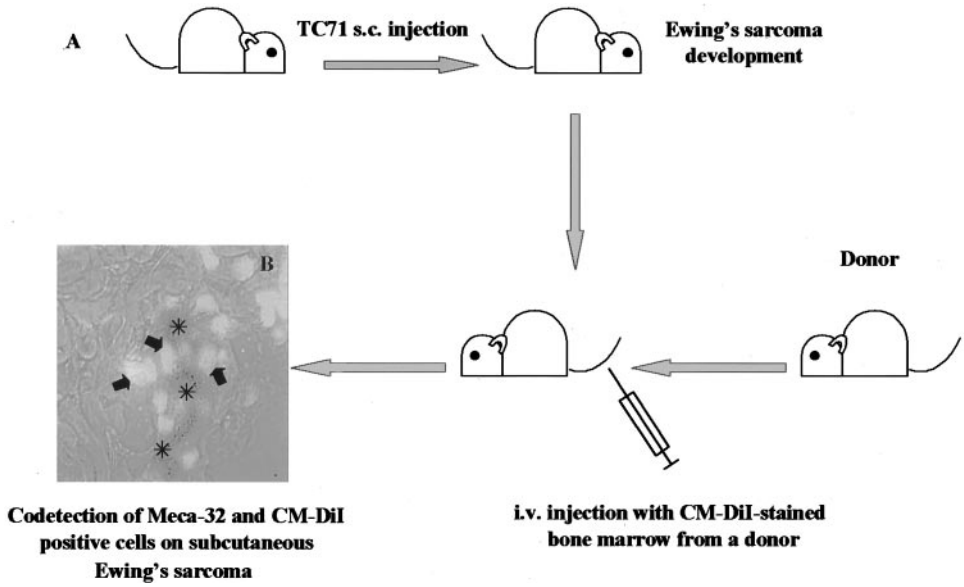

Fig. 2 Localization of donor BM cells in new tumor vasculature. *A,* CM-DiI-stained whole BM cells were injected into the tail veins of syngeneic BALB/c mice that had been inoculated s.c. with Ewing's sarcoma cells 6 days earlier. *B,* bright-field microscopy and fluorescence microscopy revealed that CM-DiI-positive cells were present close to and within Meca-32-positive vessels in the tumor (a representative panel is shown). *Full arrows* indicate CM-DiI-positive cells. *Stars* indicate a Meca-32-positive vessel.

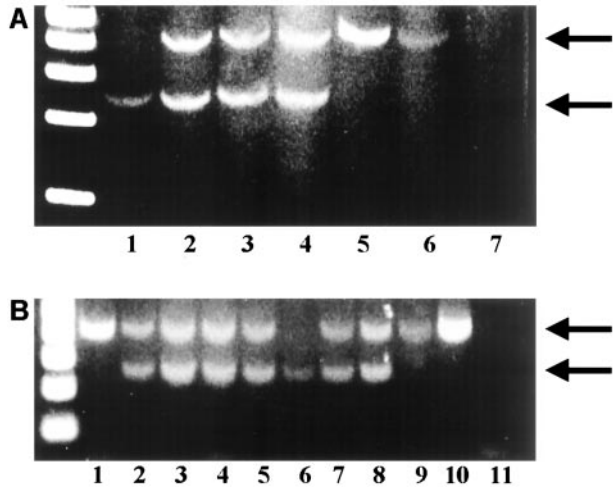

Fig. 3 Detection of IL-1β polymorphism in spleens and peripheral blood from mice who underwent BMT. The presence of both bands indicates CB6 F1 donor origin. *Top band* indicates recipient strain (BALB/c). *Bottom band* indicates donor strain (CB6 F1). *A, Lanes 1–4* correspond to individual spleens from chimeric mice. *Lane 5* corresponds to a spleen from a BALB/c mouse that did not undergo BMT (control). *Lane 6* corresponds to a control BALB/c liver. *Lane 7* corresponds to the PCR-negative control. *B, Lane 1* corresponds to peripheral blood from a BALB/c mouse. *Lanes 2–8* correspond to peripheral blood from individual chimeric mice. *Lane 9* corresponds to a syngeneic transplanted mouse. *Lane 10* is a control BALB/c liver, and *Lane 11* corresponds to the PCR-negative control.

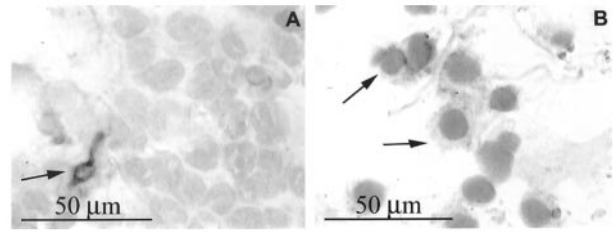

Fig. 4 Detection of donor-derived vessels in Ewing's sarcoma tumors. A BMT model exploiting MHC haplotype differences between donor and recipient mice was used to distinguish cells of donor origin (MHC b) from cells of the host (MHC d). Chimeric nude mice received s.c. injection with Ewing's sarcoma cells. I-A^b staining was used to distinguish donor BM-derived cells. *A* shows the presence of an I-A^b-positive vessel within the tumor (*arrow*). *B* shows the presence of infiltrating BM-derived I-A^b-positive cells in a Matrigel plug (*arrows*).

MHC surface antigens. Donor MHC ($H2^b$ and I-A^b) was detected in recipient spleens, documenting engraftment (data not shown).

To further confirm engraftment of the transplanted BM cells into the recipient mice, we used a strain-specific DNA polymorphism in the IL-1β gene (21). The presence of donor-origin IL-1β gene was documented in spleens and peripheral blood isolated from chimeric mice. Peripheral mononuclear cells and spleens from BALB/c mice transplanted with BM cells from CB6 F1 mice demonstrated IL-1β gene bands of two different sizes (Fig. 3). The presence of both donor and recipient IL-1β gene indicated engraftment of the CB6 F1 BM cells. BALB/c mice transplanted with syngeneic BM showed only one IL-1β gene band.

Chimeric nude mice were injected s.c. with a Ewing's sarcoma cell line (see "Materials and Methods"). Donor I-A^b-positive vessel-like structures were detected in the tumors excised from these mice (Fig. 4). These surface antigens are from donor origin. Colocalization of H-$2K^b$ and PECAM/CD31 on some tumor vessels was also seen, with the donor-positive vessels accounting for 10% of the tumor vasculature (Fig. 5). This indicates that these H-$2K^b$-positive structures are indeed vessels and that their origin is from the donor BM cells.

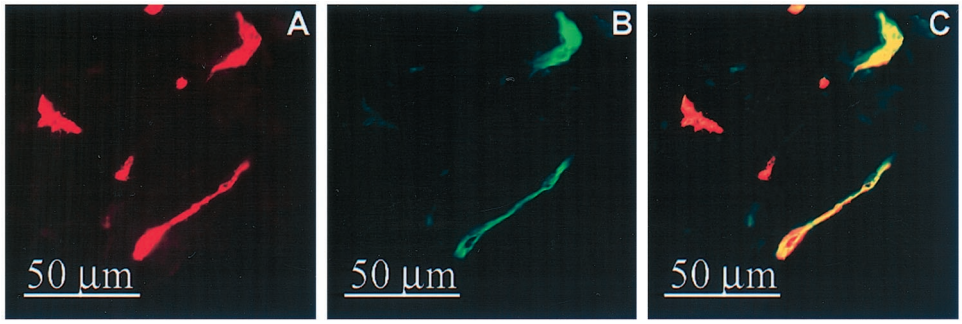

Fig. 5 Colocalization of donor BM-derived cells and CD31-positive vessels in a Ewing's sarcoma tumor from a chimeric mouse. A BMT model exploiting MHC haplotype differences between donor and recipient mice was used to distinguish cells of donor origin (MHC b) from cells of the host (MHC d). Chimeric nude mice received s.c. injection with Ewing's sarcoma cells. CD31 staining was used to distinguish vessels (*red staining*), and H-2 K^b staining was used to distinguish donor BM-derived cells (*green staining*). *A* shows CD31-positive vessels (*red staining*) on a tumor from a chimeric mouse. *B* shows some of those vessels positive for H2-K^b (donor) staining (*green*). *C* shows a superposition of both markers on the selected area (*yellow*).

DISCUSSION

Angiogenesis, the process by which new vasculature develops from the proliferation of preexisting, fully differentiated endothelial cells in the area of the tumor, plays an important role in tumor growth (22). Angiogenesis is also essential in normal hair growth, in the formation of corpus luteum during the estral cycle, in wound healing, and in diabetic retinopathy (4, 23–27). By contrast, in vasculogenesis, new blood vessels are formed from endothelial precursors, which migrate to the area of vessel development. These newly formed vessels are then remodeled into mature vessels by angiogenesis (2). Vasculogenesis was originally thought to occur only in embryonic development. However, several studies demonstrate that vasculogenesis may also be involved in postnatal life when new vascular networks are formed or expanded (7–12).

Our findings demonstrate that vasculogenesis was involved in the growth and development of Ewing's sarcoma in our mouse model. Our *in vitro* studies showed that BM cells could give rise to endothelial-like cells that expressed the markers CD105 (endoglin) and E-selectin and uptake acLDL, which are characteristics of endothelial cells. When BM cells were stained with the fluorescent cell tracker dye CM-DiI (28) and then injected i.v. into mice with Ewing's sarcoma, the cells migrated into the area of new vessel development within the tumor and colocalized with Meca-32-stained microvessels (Fig. 2). The CM-DiI-stained cells appeared close to and within the vessels. This colocalization was not seen when non-BM cells were injected, demonstrating the unique migration ability of the BM cells (data not shown). To preclude the possibility that these BM-derived cells were inflammatory cells recruited into the area of the microvasculature, we stained for Mac-1, MRP-14, and Scavenger, markers of inflammation (29–31). We have not found costaining of these markers in the areas of CM-DiI-positive structures and cells, thus excluding a nonspecific inflammatory response associated with the presence of the CM-DiI-positive cells (data not shown). Given that it is highly unlikely that 100% of the injected BM cells would be stained with CM-DiI, and given that the mice's native unstained BM cells could also participate in this process, the fact that we detected fluorescent cells in the vessels was highly suggestive of specific localization of BM-derived cells to the tumor vessel area. The appearance of these cells was very similar to the appearance of stem cell leukemia/T-cell acute leukemia-1-positive cells [SCL has been proposed as a marker of angioblasts (32)] on sites of neovascularization.

To further investigate the role of BM-derived cells in tumor vascular development, we used a mouse BMT model. We made use of differences in MHC determinants between donor and recipient mice to be able to track donor BM-derived cells after BMT. Any cells positive for donor MHC detected at remote sites could be inferred to have been derived from donor BM and delivered to those sites through the peripheral circulation.

Using our Ewing's sarcoma BALB/c nude mouse model (33), we demonstrated that donor BM-derived cells were involved in the formation of the tumor vasculature. Chimeric mice received s.c. injection of Ewing's sarcoma tumor cells. Tumors obtained from these animals showed numerous vessels positive for H-2K^b.

Colocalization of CD31 (6, 34) and H-2K^b on the tumor vessels indicated that these vessels were indeed derived from the transplanted BM cells (Fig. 5). Donor-positive vessels accounted for approximately 10% of the tumor vasculature. Together, these data suggest that BM cells contain endothelial progenitor cells that are attracted to the tumor area through chemotaxis and subsequently contribute to the vascular development needed to support the growth of Ewing's sarcoma. Both vasculogenesis and angiogenesis can thus support neovascularization within this tumor.

We have previously shown that Ewing's sarcoma cells overexpress VEGF (35). It was previously shown that VEGF can mediate the migration of human monocytes through chemotaxis (36). Based on this, it is tempting to speculate that VEGF, in addition to stimulating angiogenesis in the tumor area, may also serve to stimulate and attract the participation of BM-derived cells in the vascular development of the tumor (37). Indeed, Asahara *et al.* (38) demonstrated increased corneal neovascularization secondary to VEGF-induced mobilization of BM-derived cells. The identification of a population of cells in the BM that can localize directly to the tumor area through chemotaxis, where they home and participate in tumor vasculogenesis, opens the possibility of exploiting these cells for antitumor therapy and possibly gene therapy as well. Modulation of the vasculogenesis process may also provide new therapeutic targets.

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