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Original Article

Transplanted Bone Marrow Cells Preferentially Home To The Vessels Of In Situ Generated Murine Tumors Rather Than Of Normal Organs

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ABSTRACT

Transplanted bone marrow-derived (BM) cells have been shown to home into the tumor vessels of s.c. implanted tumor models and to functionally contribute to tumor neoangiogenesis and tumor growth. However, whether BM cells contribute to the vessels of in situ developing tumors remains unknown. We have taken advantage of the in situ generation of mammary tumors in transgenic mice carrying the polyoma virus middle T oncogene (MMTV-PyVT) to determine whether transplanted BM cells home to and incorporate into the intratumoral vessels. Unfractionated BM from lacZ+ROSA 26 mice was used to rescue irradiated MMTV-PyVT transgenic mice or their wild-type congenics. All transgenic mice were sacrificed when they developed easily palpable mammary tumors. BM cells recruited and incorporated into the vasculature were identified by coexpression of lacZ and CD31, evidence that these cells had a distinctive, elongated appearance and that they lined the vessel structures. We found that BM cells home to and incorporate into 1.3% of the vessels of all in situ generated mammary adenocarcinomas examined ($n = 8$). In contrast, BM cells did not recruit into the vessels of colon or liver of the tumor-bearing mice. Whether these cells contribute to new vessel formation via vasculogenesis or angiogenesis or simply attach to, and integrate into, the growing tips or shafts of preexisting vessels has to be determined. BM could be used as a vehicle for the specific transport of antiangiogenic signals into the tumor vascular bed. *Stem Cells 2004;22:86-92*

INTRODUCTION

The formation of new blood vessels is required for the growth and spread of tumors [1]. Bone marrow transplantation (BMT) studies in animals challenged with s.c. implanted tumors indicate that bone marrow (BM)-derived cells migrate to the tumors and incorporate into the tumor vessels [2-6]. Initial studies have suggested that these BM-derived vessel-associated cells (BM-VCs) represent BM-derived endothelial cells which participate in new vessel formation through the mechanism of vasculogenesis [2-5]. More recently, a study claimed that BM-VCs are not differentiated endothelial cells but rather hematopoietic cells that home into the tumor vasculature and, at least a subset of them promotes angiogenesis [6]. Nevertheless, these studies could demonstrate the functional role of BM cells in tumor angiogenesis. Gene manipulation or elimination of BM cells that

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home into the tumor vascular bed resulted not only in inhibition of tumor neoangiogenesis but also in impaired tumor growth [4-6].

However, because of the lack of suitable in vivo models, previous studies evaluating the presence and the role of BM cells recruited and incorporated into the tumor vasculature could not reliably evaluate the in vivo situation during tumorigenesis and tumor growth. Exogenous, s.c. implanted tumors differ from orthotopic, spontaneously in situ developed tumors in many ways such as morphology, tumor growth pattern, vessel morphology as well as response to angiogenic and antiangiogenic stimuli [7]. The tissue bed in which the exogenous tumor is implanted may also differ from the stroma in which a tumor is generated in situ. Particular characteristics of these different stroma, like blood supply, morphology and functionality of the already existing vascular bed, extracellular matrix composition and cytokine milieu may affect the recruitment of BM cells and their ability to home and incorporate into functional vessels [8-10]. Moreover, needle stab tissue injury, which is unavoidable during the s.c. implantation of the tumor, may affect the migration of BM cells in the tumor vasculature.

Female transgenic mice carrying the polyoma virus middle T oncogene under the transcriptional control of the mouse mammary tumor virus promoter (MMTV-PyVT) develop palpable mammary adenocarcinomas as early as 5 weeks of age [11]. Male transgene carriers also develop adenocarcinomas at later time points. We have taken advantage of the in situ generation of tumors in this transgenic mouse model to determine whether BM-derived cells preferentially and substantially home to and incorporate into the tumor vessels. This information is essential before one uses BM as a vehicle for transport of anti-angiogenetic signals into the tumor vascular bed.

Materials and Methods

BMT

FVB-TgN(MMTV-PyVT) transgenic mice (The Jackson Laboratory; Bar Harbor, ME; http://www.jax.org) 3-4 weeks of age or their age-matched wild-type (WT) congenics FVB were lethally irradiated with 8.5 Gray of a ^{137}Cs gamma-source. Mice were rescued by intravenously administrated unfractioned BM cells ($2\text{-}5 \times 10^6$ cells) obtained aseptically by flushing the tibias and femurs of age-matched transgenic mice constitutively expressing lacZ in all tissues (TgR(ROSA 26)26 Sor (The Jackson Laboratory). In some cases, ROSA 26 BM donors were treated with vascular endothelial growth factor (VEGF) (80 ng/100 μl phosphate-buffered saline [PBS], recombinant mouse VEGF, [R&D Systems; Minneapolis, MN; http://www.rndsystems.com]) delivered i.p. once a day for 5 days before the BM was harvested. MMTV-PyVT mice were sacrificed when they developed easily palpable tumors. FVB controls were sacrificed either at the same time points, or were observed for periods of up to 600 days post-transplantation. Hematopoietic engraftment was assessed by fluorescence-activated cell sorter (FACS) analysis of retro-orbital-collected blood samples or BM with antibodies that react with the H-2Kb major histocompatibility complex (MHC) class I alloantigen (clone AF6-88.5, phycoerythrin [PE] conjugated) (BD Biosciences; San Diego, CA; http://www.biosciences.com), which recognizes cells from the ROSA 26 mice (C57BL6 background strain) and with antibodies specific for the H-2Kq MHC class I alloantigen (clone KH114, fluorescein isothiocyanate [FITC] conjugated), (BD Biosciences) which recognize cells from MMTV-PyVT mice (FVB background strain). These experiments were performed in accordance with a protocol approved by the local Institutional Animal Care and Use Committee (Regierungspräsidium Freiburg, Protocol Nr: AZ 35-9185.81/1/288.1).

Immunohistochemical Analysis

Animals were perfused transcardially with PBS to minimize blood cell contamination before organs and tumors were excised. Frozen sections (5 μm) were fixed in cold methanol/acetone solution (1:1) for 10 minutes, and air dried. For lacZ staining, samples were washed three times in PBS and incubated overnight at 37°C in PBS containing 1% 5-bromo-4-chloro-3-indolyl-N-acetyl-β-D-galactopyradoside (X-Gal Solution, [Sigma; St. Louis, MO; http://www.sigmaaldrich.com]), 5 mM potassium ferricyanide, 5mM potassium ferrocyanide, and 2 mM $MgCl_2$. The tissues were then washed in PBS and immunostained with rat anti-mouse CD31 (clone MEC13.3, (Pharmingen Europe; http://www.bdbiosciences.com/pharmingen) or CD45 (clone CBL 1321, Cymbus Biotechnology LTD; Hampshire, UK; http://www.chemicon.com) specific monoclonal antibodies. Antibody binding was detected with diaminobenzidine chromogen (DAB) by using the InnoGenex™ iso-immunohistochemistry Kit (InnoGenex; San Ramon, CA; http://www.innogenex.com). Briefly, sections were blocked for non-specific antibody binding with protein blocking reagent PowerBlock™ (InnoGenex), incubated with a rat anti-mouse CD31 or CD45 specific monoclonal antibody, washed in PBS, and then incubated with biotinylated goat anti-rat immunglobulin (InnoGenex). The bound biotin-labeled antibody was then detected by the serial addition of horseradish peroxidase-streptavidin conjugate and DAB substrate according to the manufacturer's instructions. The specificity of peroxidase reaction was confirmed by the absence of staining when unspecific rat immunoglobulins (DAKO; Glostrup, Denmark;

http://www.blackwell-synergy.com) were used instead of the primary antibody. Photomicrographs were taken with Zeiss Axioplan 2 Imaging and Axion Vision 3. Differences in vessel density were evaluated by enumeration of $CD31^+$ structures/low power field (20× magnification). Incorporation of BM cells into the vessels was determined by two independent, blinded, observers (*M.M.* and *A.S.*) by using strict criteria (**Results**). Approximately 300 vessel profiles were analyzed per tumor, colon, or liver section.

Results

Unfractionated BM from ROSA 26 mice was used as a hematopoietic graft to rescue irradiated MMTV-PyVT transgenic mice (n = 12) or their WT congenics FVB (n = 21). At transplantation all recipients were between 3 to 4 weeks in age and had no palpable tumors. Two transgenic and four WT mice died within a median of 20 days after transplantation, probably due to infections or engraftment failure. No clinical signs of graft-versus-host-reactions to skin were observed. All evaluable MMTV-PyVT mice (n = 10) developed tumors in the mammary pad that histologically proved to be adenocarcinomas. Female MMTV-PyVT recipients (n = 6) developed palpable tumors in a median of 60 days after BMT (range 46-65), whereas male recipients (n = 6) exhibited palpable tumors in a median of 192 days after transplantation (range 159-415). Counting from the day of birth, all MMTV-PyVT BM recipients developed tumors at time points similar to their nontransplanted female and male counterparts, indicating that the irradiation and the BMT had no influence on tumor cell development and growth. MMTV-PyVT mice were sacrificed when the tumors were easily palpable. FVB controls were sacrificed either at the same time points, or were observed for periods of up to 600 days post-transplantation. Complete autopsies revealed no tumor formation in the FVB mice at any time point after BMT. All transplanted mice, including those followed up to 1 year after transplantation, revealed a stable >80% hematopoietic engraftment as determined by FACS enumeration of $H\text{-}2Kb^+$ cells (ROSA 26 specific MHC class I allele) in retro-orbital blood samples or BM samples (Fig. 1).

To assess whether BM cells were recruited to the tumors and incorporated into the vessels, we performed double stains with enzymatic lacZ stain followed by immunohistochemistry with anti-mouse CD31 antibodies. The specificity and sensitivity of the lacZ/CD31 stain were tested in organ biopsies of ROSA 26 mice and FVB or MMTV-PyVT nontransplanted mice (Fig. 2). In the ROSA 26 mice all cells from aorta tissue as well as colon or liver sections stained blue. In contrast, blue stain was not seen in colon and liver biopsies from FVB or MMTV-PyVT mice. The immunohistochemical stain with anti-CD31 antibodies established the presence of vessel structures with typical morphologies in colon, liver and in higher densities in the MMTV-PyVT tumors. The intratumoral vessel density was not different in the transplanted versus the nontransplanted MMTV-PyVT mice. We used strict criteria for the identification of BMVCs that homed and incorporated into the tumor vasculature. These included co-staining of lacZ and CD31, strong CD31 immunostaining, distinctive elongated morphology, presence of the double-stained cell in line with a structure having typical vessel morphology, and absence of tumor necrosis in the area of examination. Since tumor necrosis areas revealed a large number of $lacZ^+$ hematopoietic cells, they were excluded from analysis. In contrast, $lacZ^+$ cells were only rarely seen in non-necrotic areas (Fig. 2E). Donor-derived cells were evaluated for co-expression of the pan-hematopoietic marker CD45. $LacZ^+$ cells found to line structures with typical vessel morphology were uniformly CD45 negative,

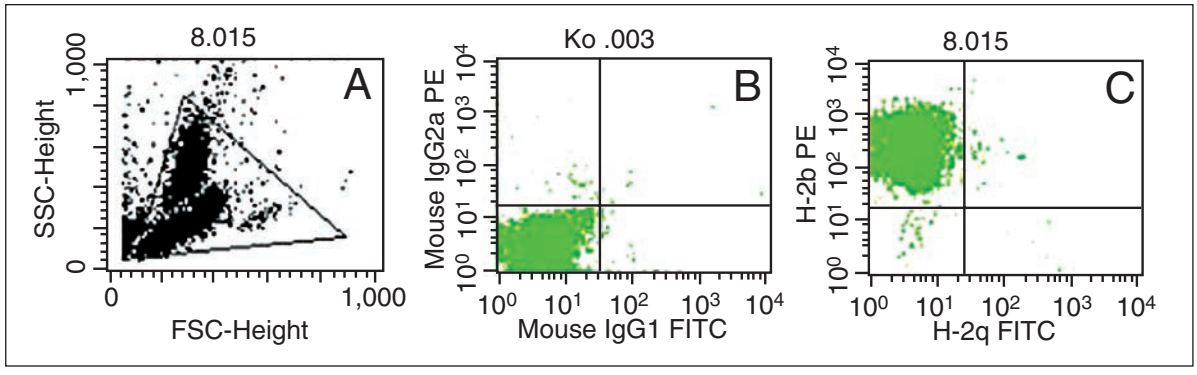

Figure 1. Hematopoietic engraftment after allogenic BMT in mice. *FACS analysis of peripheral blood from a representative MMTV-PyVT mouse (FVB strain) tranplanted with unfractionated BM harvested from ROSA 26 mice (C57BL6 strain). A) The analysis gate used to exclude dead cells and debris. B) Negative control. C) Incubation of blood cells with an antibody against H-2b PE (which recognizes C57BL6 strain) and with an antibody against H-2q FITC (which recognizes FVB strain) showing hematopoietic engraftment greater than 90%.*

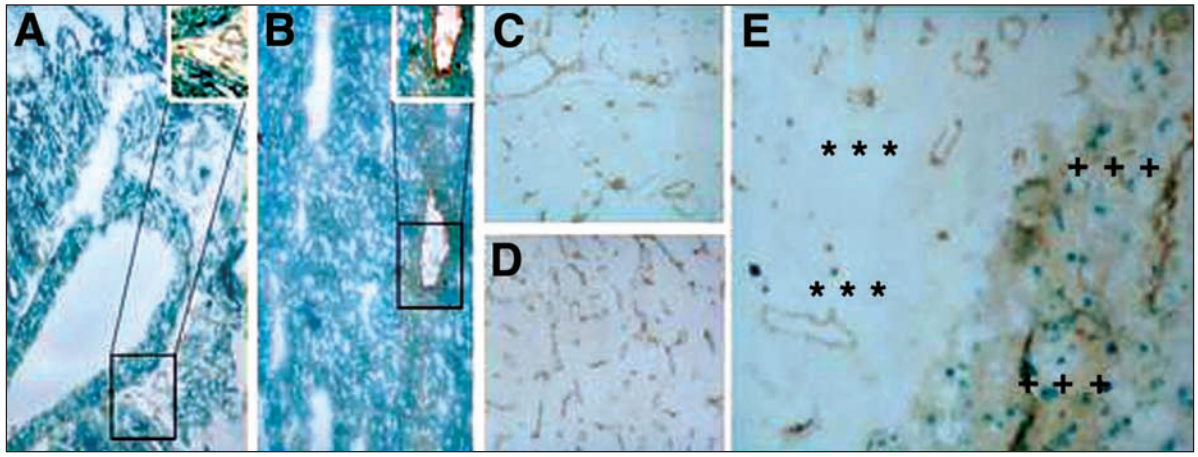

Figure 2. Sensitivity and specifity of the double lacZ stain/CD31 stain. *A, B) Positive controls: colon (A) and liver (B) section of a ROSA 26 mouse. All cells stained blue while the endothelial cells co-stained with CD31. C, D) Negative controls: All cells of colon tissue (C) or mammary tumors (D) from MMTV-PyVT mice were stained negative for lacZ while the CD31 stain revealed the vessel structures. E) Tumors developed in MMTV-PyVT mice previously transplanted with lacZ$^+$ whole BM. Tumor necrosis areas (+++) revealing a large number of lacZ$^+$ hematopoietic cells were excluded from analysis. In contrast, lacZ$^+$ cells were only rarely seen in non-necrotic areas (***). Original magnification, ×20.*

although the CD45 antibodies strongly stained the lacZ$^+$ cells within the necrotic areas. Therefore, no triple CD45/CD31/lacZ stain was performed.

In order to estimate the degree of recruitment and incorporation of BM cells into the tumoral vessels, approximately 300 vessel profiles were analyzed per tumor tissue section. Approximately 1.3% of the tumor vessel walls were found to contain lacZ$^+$/CD31$^+$ BM-VCs (Table 1). In most of the cases, only one lacZ$^+$/CD31$^+$ cell was found incorporated into the vessel (Fig. 3). However, in some cases a more robust incorporation of BM cells in the tumor vessel was observed (Fig. 3C).

We treated ROSA 26 BM donors with VEGF delivered i.p. for 5 days before we harvested the BM cells. Four female MMTV-PyVT mice were transplanted with VEGF-conditioned BM. Palpable tumors developed with a median of 74 days post-transplantation, which did not differ from the tumor development observed in the other MMTV-PyVT female mice. BM cell recruitment and incorporation into the tumor vasculature of the mice transplanted with VEGF-conditioned BM were at a degree similar to that found in the MMTV-PyVT mice receiving non-conditioned BM graft (Table 1).

The incorporation of BM cells into vessels outside the tumor was tested in colon and liver sections from the 14 MMTV-PyVT mice transplanted with BM harvested from VEGF-treated and nontreated ROSA 26 donors (Table 1). No lacZ$^+$/CD31$^+$ cells were found in any of the tissue sections of any of the mice examined. In addition, mice sacrificed at later time points after transplantation (~ 300 days) did not show any incorporation of BM cells into the extratumoral vessels.

Discussion

Delivering antiangiogenic signals specifically into tumors is a promising anticancer strategy [1, 12]. The central finding of this study is that transplanted BM cells recruit and home into the vessels of in situ-generated tumors, but not into the vessels of normal organs. Donor-derived cells incorporated into the tumor vessel structures were stained positive for CD31 and exhibited an elongated appearance. In contrast, lacZ$^+$ cells found to be integrated into the surface of structures with typical vessel morphology were uniformly CD45 negative. These characteristics are strongly indicative of endothelial cells. However, since we did not use confocal microscopy for tissue examination, we cannot rule out the possibility that these lacZ$^+$/CD31$^+$ cells lining the tumor vessels are not differentiated endothelial cells, but are rather hematopoietic in nature. Indeed, a recent report suggests that transplanted BM cells found incorporated into the vessels were more likely to represent hematopoietic cells with angiogenic properties than BM-derived endothelial cells [6]. The BM-derived cells we described here and which were found to be integrated into the tumor vessels probably include BM-derived endothelial cells, the recently reported Tie-2-expressing hematopoietic cells with angiogenic activities, and very likely also leukocytes or macrophages bound to the endothelium or in diapedesis as well. Nevertheless, these BM-VCs homed preferentially into the tumor vasculature rather than into the vessels of normal organs like the colon or liver. Thus, specific stimuli in tumors recruit and augment the incorporation of BM cells in the vasculature. Since we did not perform tissue injuries with establishment of sites of new vessel formation in our mouse model, we cannot define

Table 1. Incorporation of BM cells into the vasculature of tumor-bearing mice

MMTV-PyVT mouse number	Sex	Type of ROSA 26 BM graft	Time point of analysis (days after BMT)	Positive intratumoral vessels (numbers/ total)[a]	% involvement of BM cells to vasculature[b]		
					Tumor	Colon	Liver
245	f	bm	46	4/380	1.0%	0	0
248	f	bm	46	5/330	1.5%	0	0
244	f	bm	60	3/213	1.4%	0	0
246	f	bm	60	3/470	0.6%	0	0
9	m	bm	159	2/103	1.9%	0	0
2	m	bm	242	3/383	0.8%	0	0
332	f	bm	65	ne	ne	0	0
351	m	bm	172	6/303	1.9%	0	0
355	m	bm	172	6/331	1.8%	0	0
354	m	bm	294	ne	ne	0	0
350	f	VEGF-bm	110	4/310	1.3%	0	0
368	f	VEGF-bm	74	3/290	1.0%	0	0
369	f	VEGF-bm	74	3/345	0.9%	0	0
370	f	VEGF-bm	74	5/360	1.4%	0	0
				Mean:	1.3%	0	

Irradiated, 3-4-week-old MMTV-PyVT transgenic mice (f: female, m: male) were transplanted with unfractionated bone marrow (BM) harvested from either untreated ROSA 26 mice (bm) or ROSA 26 mice previously treated for 5 days with i.p. VEGF (VEGF-bm). Mice were sacrificed when they developed easily palpable tumors, at the time points indicated. BM-derived cells which had incorporated into the vessels of tumor, colon, or liver were identified by lacZ/CD31 stain as indicated in the **Results** section (BM-vessel-associated cells, BM-VC).

[a]Tumor vessels were considered positive when they contained one or more BM-VC.

[b]The mean contribution of BM cells to the vasculature is estimated by evaluation of the proportion of positive tumor vessels relative to approximately 300 analyzed vessels.

ne = not evaluated.

whether the differences observed between tumor and organ vessels are related to the high vessel generation seen in tumors or are related to tumor-elaborated factors.

Studies evaluating the homing of BM cells in tumor vessels have been hampered by the lack of suitable in vivo models. All animal studies performed up to now used s.c.

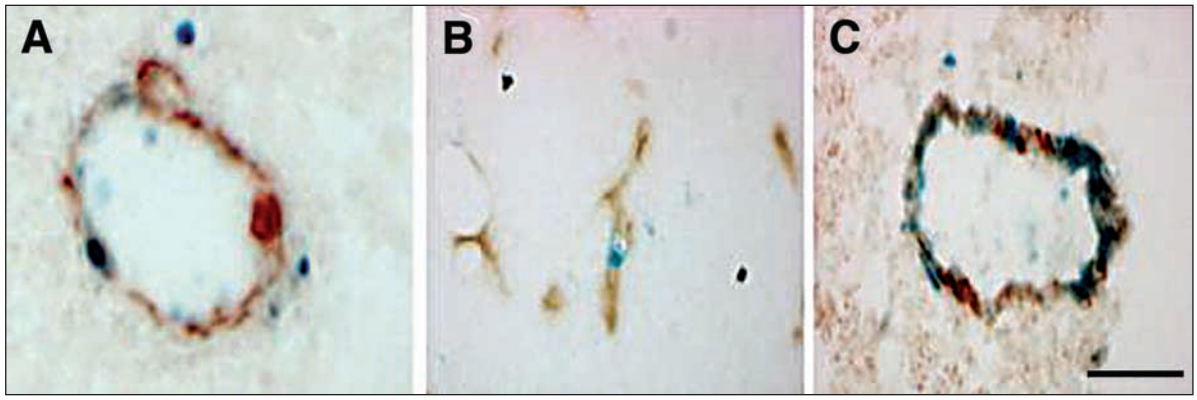

Figure 3. Incorporation of BM-derived cells into the intratumoral vessels. *lacZ/CD31 double staining of tumors developed in MMTV-PyVT mice previously transplanted with lacZ$^+$ BM. In most of the cases, only one lacZ$^+$/CD31$^+$ cell was found incorporated into the vessel (A, B). However, in some cases a more robust incorporation of BM cells in the tumor vessel was observed (C). Scale bar, 20 μm.*

implanted tumor models [2-6]. It has been shown that tumor angiogenesis in s.c. tumors may differ from those of orthotopic developed tumors in various characteristics such as morphology, functionality, and involvement of angiogenic factors [7]. The implantation area of the tumor cells may differ in many ways from the specific stroma in which a tumor generates and grows in situ [8-10]. Differences in blood supply, functionality, and morphology of the preexisting vascular bed, extracellular matrix constitution and cytokine milieu may alter the ability of BM cells to reach and incorporate into the new vessels. Moreover, implantation of tumor cells in s.c. tissue is obligatorily combined with tissue injury due to the needle stab. Whether BM cell recruitment and incorporation into the vasculature of s.c. implanted tumors is attributed to tumor- or injury-specific signals could not be excluded in previous studies. In situ generated tumors in transgenic mice most reliably resemble the in vivo situation of tumorigenesis and tumor growth. Compared to s.c.-implanted tumors, mammary tumors in the MMTV-PyVT mice grow in the stroma of the mammary gland and not in a foreign stroma. The MMTV-PyVT tumors show a progression from in situ carcinomas which consist of only a few tumor cells to invasive palpable tumors. This is in comparison to s.c. tumors which at implantation consist of millions of cells. At last, needle stab injury during s.c. tumor implantation is excluded as an exogenous factor influencing BM cell incorporation into the vessels of MMTV-PyVT-developing tumors. Therefore, our data confirm previous notions that BM cells may home and incorporate specifically into tumor vessels and extend these findings by excluding effects attributable to experimental design.

We found recruitment of BM cells into the tumor vessels of all mice studied. However, the degree of incorporation was below 2% in both the tumors that developed early after BMT as well as those that developed later. The more robust contribution of BM cells in the tumor vascular bed reported in previous animal studies is probably related to the different experimental approaches used. BM cells were found to be incorporated in up to 90% of new vessels in tumors s.c. grown in angiogenesis defective Id-mutant mice [5]. Endothelial cells generated and expanded ex vivo from mesenchymal BM cells were found in 35% of the tumor vessels when given i.v. in conditioned, s.c. tumor mice models [3]. *Davidoff et al*. [4] found about 5% green florescent protein (GFP) expressing $CD31^+$ endothelial cells in the vessels of neuroblastoma grown in the s.c. tissue of mice already underwent transplantation with GFP-transduced BM cells. Variations of the experimental settings between these reports and our study, such as s.c. versus in situ tumor growth, congenic versus allogeneic BM transplantation, presence or absence of angiogenesis defects in the transplanted mice, lacZ versus GFP detection techniques of donor cells, different BM fractions transplanted or different time points of analysis post-BMT are only some of the points which could reflect the differences reported in the degree of recruitment and incorporation of BM cells in the tumor vascular bed. Nevertheless, ours and previous studies suggest that recruitment of BM cells was restricted to the tumor vascular bed and not to sites of nontumor angiogenesis.

The experiments described here do not determine which cell(s) within the BM actually give rise to the BM-VCs. Since tumors developed with a latent period of several weeks after BMT, integration of BM cells in the tumor vessels was not caused by lodging of donor-derived cells at the time of i.v. injection but most likely by endogenous seeding of cells from the engrafted BM. Male mice developed tumors at late time points after transplantation (approximately 300 days) and showed a similar incorporation of BM cells into the tumor vessels as seen in female mice sacrificed earlier. Therefore, it seems very likely that BM-VCs derive from precursors with long-term engraftment.

Both transplanted and nontransplanted MMTV-PyVT mice showed the same intratumoral vessel density and the same growth kinetics for tumor development. Since our experimental design did not include manipulation of the BM graft in order to use it as a vehicle of antiangiogenic targets, we cannot formally answer the question of whether or not the BM-VCs described here play a significant role in tumor angiogenesis and tumor growth. The latter seems very unlikely because of their low frequency (<2%). However, *Davidoff et al*. [4] could influence angiogenesis and growth of s.c. tumors in mice by delivering antiangiogenic signals via BM transplantation, although the contribution of BM-derived cells in the tumor vasculature was found to be no more than 5%. Additionaly, *De Palma et al*. [6] could achieve substantial inhibition of angiogenesis and impairment of tumor growth by elimination of a subset of BM-derived cells that homed and incorporated into the tumor vessels.

VEGF is a key regulator of physiological and pathological angiogenesis and has been shown to exert pleiotropic effects on both endothelial cells and BM cells [13]. In vitro, VEGF has been shown to induce differentiation of BM mesenchymal cells into endothelial cells [3], to increase proliferation of endothelial progenitor cells [14], and to promote chemotaxis of BM mononuclear cells [14, 15]. VEGF delivery to adult mice has been shown to increase BM-derived endothelial cells in blood [14] as well as to increase the generation of immature myeloid stem cells in the BM [16]. We conditioned the ROSA 26 donor mice for 5 days with i.p. delivered VEGF before we harvested the BM. We preferred to treat the donor mice with VEGF and

not the recipient tumor-bearing mice in order to simulate a possible therapeutic maneuver with ex vivo gene-engineered BM. VEGF application in BM donors compared to tumor patients does not encounter ethical problems related to the mitogenic and tumor-growth-promoting effects of VEGF [13]. Since we did not perform FACS analysis of the harvested BM, we cannot conclude if this treatment altered the BM cellularity or cell composition of the BM graft compared to the BM graft obtained from nontreated mice. Nevertheless, this in vivo graft manipulation did not increase the proportion of BM cells recruited and incorporated into the vessels of the MMTV-PyVT tumors. The large number of hematopoietic cells in the tumor necrosis areas could be either due to chemoattractants, like tumor necrosis factor-α or factors of the interleukin-6 cytokine family, or due to leakage from destroyed vessels. No differences in the number of lacZ$^+$ cells in the necrosis areas between mice transplanted with VEGF-conditioned or nonconditioned BM were observed, as evaluated by enumeration of lacZ$^+$ cells/low power field (20× magnification).

We conclude that transplanted BM cells recruit and home into the tumor vascular bed of in situ generated murine tumors but not into normal tissue. Whether these cells contribute to new vessel formation via vasculogenesis or angiogenesis or simply attach to and integrate into the growing tips or shafts of pre-existing vessels has to be determined. The understanding of the factors that promote the recruitment and incorporation of BM cells into the tumor vasculature may elucidate the development of anticancer gene therapies in which ex vivo manipulated BM may be used as a vehicle for the specific transport of antiangiogenic signals into the tumor vascular bed.

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