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Vascular Progenitors Derived From Murine Bone Marrow Stromal Cells Are Regulated by Fibroblast Growth Factor and Are Avidly Recruited by Vascularizing Tumors

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Abstract Bone marrow-derived stromal cells (BMSC) possess a population of vascular progenitor cells that enable them to acquire a histology and immunophenotype coherent with endothelial cells (EC). Recent evidence indicates that a hypoxic environment such as that encountered in tumor masses regulates BMSC angiogenic properties by pathways that remain to be defined. It is also unclear as to what extent these marrow-derived precursor cells could contribute to the growth of endothelium-lined vessels at the vicinity of tumor masses. In this study, we found that BMSC exhibited the ability to generate three-dimensional capillary-like networks on Matrigel, and that this property was up-regulated by growth factors-enriched conditioned media isolated from several tumor-derived cell lines. In particular, basic fibroblast growth factor, a key mediator of angiogenesis, was found to be the most potent growth factor for inducing BMSC proliferation, migration, and tubulogenesis. The setup of a new two-dimensional in vitro co-culture assay further showed that BMSC were massively recruited when cultured in the presence of either cancerous or differentiated EC lines. In vivo, subcutaneous co-injection of BMSC with U-87 glioma cells in nude mice resulted in the formation of highly vascularized tumors, where BMSC differentiated into CD31-positive cells and localized at the lumen of vascular structures. Our data suggest that BMSC could be recruited at the sites of active tumor neovascularization through paracrine regulation of their angiogenic properties. These observations may have crucial implications in the development of novel therapies using BMSC engineered to secrete anti-cancerous agents and to antagonize tumor progression. J. Cell. Biochem. 91: 1146–1158, 2004.

Key words: mesenchymal stem cells; tumor angiogenesis; growth factors

Bone marrow-derived stromal cells (BMSC) represent a subpopulation of non-hematopoietic pluripotent cells within the bone marrow microenvironment and are frequently referred to as mesenchymal stem cells due to their ability to differentiate into many mesenchymal phenotypes [Caplan, 1991; Prockop, 1997]. In contrast to their hematopoietic counterparts, BMSC demonstrate a strikingly enhanced ability to adhere to tissue-culture surfaces and to differentiate in culture into osteogenic, chondrogenic, tendonogenic, adipogenic, and myogenic lineages [Majumdar et al., 1998]. The potential of mesenchyme-derived BMSC has further gained increased attention, since these cells were even reported to differentiate into cells of non-mesenchymal origin such as myocytes [Makino et al., 1999] and neuron-like cells [Woodbury et al., 2000]. Another type of bone marrow-derived stem cell, termed multipotent adult progenitor cell (MAPC), has also been described to give rise to other lineages as different as endothelium, endoderm, and ectoderm [Reyes et al., 2001, 2002]. However, these cells differ from BMSC in the expression of CD44 and

Abbreviations used: BAEC, bovine aortic endothelial cells; EC, endothelial cells; ECM, extracellular matrix; bFGF, basic fibroblast growth factor; HMEC, human microvascular endothelial cells; hpf, high-power field; MMP, matrix metalloproteinase; BMSC, bone marrow-derived stromal cells; S1P, sphingosine-1-phosphate; VEGF, vascular endothelial growth factor; BV, blood vessels.

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previous studies have reported an overlapping, but not identical, differentiation potential. Since the cell culture conditions of BMSC and MAPC are substantially different, a comparison of these cells under identical culture conditions has yet to be performed.

BMSC appear as fusiform fibroblasts which, in their undifferentiated state, express α-smooth muscle actin, VCAM-1, CD44, and vWF, but do not express hematopoietic or endothelial cell (EC) surface markers such as CD11, CD14, CD31, CD34, CD45, and CD56 [Conget and Minguell, 1999]. Intriguingly, several studies, however, showed that peripheral blood-derived, plastic adherent CD34-negative hematopoietic stem cells possessed BMSC characteristics and were capable of producing EC, thus highlighting a potential role of BMSC in neo-vascularization [Huss et al., 2000; Tomanek and Schatteman, 2000]. Accordingly, BMSC's ability to retain their in vivo progenitor cell potential was recently demonstrated by their transdifferentiation into EC-like cells and to participate in functional vasculature structures [Eliopoulos et al., 2002; Al-Khaldi et al., 2003a]. Endothelial differentiation was also observed in other cell lines in which, for instance, the ability of monocytes to differentiate into endothelium-like cells was demonstrated [Fernandez Pujol et al., 2001; Schmeisser et al., 2001]. Recent developments have indeed provided further evidence for a potential monocytic derivation of peripheral blood-derived circulating angiogenic cells [Rehman et al., 2003].

Proper formation of blood vessels (BV) in angiogenesis is vital for the delivery of oxygen, nutrients, and growth factors essential for development, reproduction, and wound healing. It is also well established that, when derailed, angiogenesis contributes to numerous threatening disorders such as cancer. While research has generally been focusing on the two main vascular cell types, endothelial and smooth muscle cells, recent evidence indicates that bone marrow-derived stem cells may also contribute to angiogenesis, both in the embryo and the adult [Huss et al., 2000; Davidoff et al., 2001]. Interestingly, we have recently reported that BMSC implantation induced a neovascular response resulting in a significant increase in blood flow in a chronic limb ischemia model [Al-Khaldi et al., 2003b], and the ability to effectively recruit and participate in angiogenesis and arteriogenesis de novo [Al-Khaldi et al., 2003a]. Since vascular endothelial growth factor (VEGF) plays an important role in vasculogenesis and angiogenesis, and since the BMSC starting population does not express neither VEGFR-2 nor VEGFR-3, this suggests that other factors and additional receptors may ultimately regulate BMSC contribution to neovascularization. The above mentioned interest in BMSC contributing to angiogenesis is further strengthened by their recently reported selective interaction with epithelial tumor cells [Hombauer and Minguell, 2000], and by their preferential engraftment within tumors [Wallace et al., 2001; Studeny et al., 2002]. More recently, we demonstrated that a hypoxic environment such as that encountered in tumor masses regulated BMSC angiogenic properties [Annabi et al., 2003a]. Whether BMSC's angiogenic properties enable them to participate in extracellular matrix (ECM) proteolysis in order to migrate, proliferate and form capillary-like structures locally, such as within the bone marrow environment or at a distant tumor site also remains to be seen.

In this report, we provide evidence that BMSC possess the ability to form in vitro capillary-like networks. More importantly, we show that this property can be modulated through growth factors-mediated paracrine regulation by cancer and EC, with basic fibroblast growth factor (bFGF) being likely responsible for the BMSC capillary-like structure formation ability in vitro. We also show that BMSC contribute to increased neo-vascularization of tumors in vivo, and to localize to some extent within the lumen of BV. This is also supported in vitro by cancer and EC-regulated recruitment of BMSC. As BMSC are now used very effectively in cell and gene therapy for a wide spectrum of applications [Jorgensen et al., 2003; Tuan et al., 2003], their paracrine regulation at sites of vascular remodeling suggests a crucial role that bioengineered primary BMSC may provide for gene therapy purposes against tumor progression.

MATERIALS AND METHODS

Materials

Gelatin and sphingosine-1-phosphate (S1P) were purchased from Sigma (St. Louis, MO), Matrigel was from Becton Dickinson Labware (Bedford, MA), bFGF (FGF-2) and human

recombinant VEGF were from R&D Systems (Minneapolis, OH).

Cell Culture and Media

All cell lines were purchased from American Type Culture Collection. The human U-87 glioblastoma, HT-1080, and NIH 3T3 cell lines were maintained in modified Eagle's medium (MEM), while COS-7, bovine aortic endothelial (BAE), and Caki-1 cells were cultured in Dulbecco's MEM. Culture media contained 10% fetal bovine serum (HyClone Laboratories, Logan, UT), 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and cells were cultured under a 5% CO_2 atmosphere. Mouse BMSC were extracted and cultured as follows. Bone marrow was hydrostatically expelled from the femoral and/or tibial bones of C57B1/6 female mice (Charles River Laboratory, Laprairie, Que.), and collected in high-glucose DMEM supplemented with 10% fetal bovine serum and antibiotics (50 U/ml penicillin G and 50 μg/ml streptomycin from Winsent, Inc.). Bone marrow cells were then plated on tissue culture dishes in the same medium, and incubated at 37°C with 5% CO_2. The non-adherent hematopoietic cells were discarded 5 days later and the media was replaced once a week. To prevent the stromal cells from differentiating, each primary culture was replated to new plates when cell density reached 80% confluency after seeding. Our culture expanded murine BMSC were negative for FLK1/KDR (VEGF-R2), FLT-4 (VEGF-R3), Tie-2 (angiopoietin receptor), CD31, and CD45 in vitro [Eliopoulos et al., 2002; Al-Khaldi et al., 2003a], consistent with other reports [Javazon et al., 2001; Zhao et al., 2002]. Moreover, we have found that our expanded marrow-derived stromal cells expressed CD44, a marker consistent with BMSC [Conget and Minguell, 1999] yet distinct from MAPC [Reyes et al., 2001]. The sum of these phenotypic characteristics thus distinguishes our BMSC cultures from endothelial progenitor cells [Asahara et al., 1999; Quirici et al., 2001], endothelial precursor cells [Murohara et al., 2000] and angioblasts [Kocher et al., 2001]. Finally, trypsin (0.05% w.v./0.53 mM EDTA) (Invitrogen, Burlington, ON) was used for releasing the cells from the plates [Eliopoulos et al., 2002]. In our experiments, the angiogenic properties of BMSC are compared to those of an established EC line, human microvascular endothelial cells (HMEC). In addition, a retroviral vector encoding the green fluorescent protein (*GFP*) gene was stably transduced in BMSC [Eliopoulos and Galipeau, 2002]. The expression of GFP was shown not to interfere with BMSC cellular functions or differentiation potential.

Cell Migration and Proliferation Assays

Cells were dislodged after brief trypsinization and dispersed into homogeneous single cell suspensions that were washed extensively then resuspended in DMEM at a concentration of 10^6 cells/ml. To assess migration from established monolayers, cells (10^5) were dispersed onto 0.5% gelatin/PBS-coated chemotaxis filters (Costar, Amherst, MA; 8-μm pore size) within Boyden chamber inserts and allowed to adhere for 1 h at 37°C, after which they were challenged by the addition of 600 μl of a chemoattractant solution to the lower compartments. Migration was allowed to proceed for 3 h at 37°C in 5% CO_2/95% air. Cells remaining attached to the upper surface of the filters were carefully removed with a cotton swab. Cells that had migrated to the lower surface of the filters were fixed with formaldehyde, 0.1% crystal violet/20% methanol, and counted by microscopic examination. The average number of migrating cells per field was assessed by counting at least four random fields per filter. Data points indicate the mean value obtained from three separate chambers within one representative experiment. BMSC proliferation was assessed using a cell metabolic reagent assay (WST-1; Roche Diagnostics Canada, Laval, QC) for the microtiter tetrazolium assay. After a 1-h incubation at 37°C with WST-1 labeling reagent, the number of living cells was determined by measuring the ratio between absorbance at 450 and 610 nm.

Capillary-Like Structure Formation Assay

Matrigel was thawed on ice to prevent premature polymerization; aliquots of 50 μl were plated into individual wells of 96-well tissue culture plates (Costar) and allowed to polymerize at 37°C for at least 30 min. Depletion of the Matrigel was performed by replacing the media overlaying the Matrigel every 12 h by fresh serum-free media for 7 consecutive days. Soluble Matrigel-derived matrix metalloproteinase (MMP)-2 activity and VEGF content were found to be absent after depletion (Fig. 3A). Cells were removed from confluent cultures by treatment with Trypsin–EDTA. The cells were washed in serum-containing medium, then resuspended at 10^6 cells/ml. Cell suspension (100 μl) was

added to each well, with or without additional test substances, and incubated for 16–18 h at 37°C. For quantitation of tube formation, the total tube lengths from five randomly chosen areas was measured and analyzed using the Northern Eclipse image analysis software (Version 6.0; EMPIX, Inc.).

Two-Dimensional In Vitro Co-Culture Assay

BMSC engineered to stably express GFP were co-cultured in the presence of several other cell lines according to a modified procedure described by Kozien et al. [2000]. Briefly, 6-well cell culture plates were completely covered with 1% low-melt agarose dissolved in serum-free DMEM. After the agarose gelled, the right half of the agarose was physically removed; cancerous, endothelial, or non-endothelial cells were then plated and grown in their respective media until confluency. The cells adhered to and spread on the bare area and did not migrate under the remaining agarose. The left agarose gel half was then partially removed, leaving a thin agarose barrier in the middle of the well. GFP^+–BMSC (10,000 cells/cm^2) were then seeded and left to adhere and divide for 24 h. The small agarose barrier was then removed and the well was covered with BMSC culture media. Co-culture continued for 96 h at 37°C. Green fluorescent BMSC that had migrated towards the co-cultured cells were then visualized by fluorescent microscopy. A scheme summarizing the assay is shown (Fig. 1).

Growth of Solid Tumors in Mice

Female athymic nude mice (CD-1 nu/nu) (8–10 weeks old) were purchased from Charles River Laboratory. The mice were used in accordance with institutional guidelines following the approved protocols. GFP^+–BMSC and U-87 cells (10^6/mouse) were injected s.c. as a suspension in 100 μl of sterile PBS into the dorsal midline. Tumors were left to develop for 3 weeks, and mice were deeply anaesthetized with 80 mg/kg i.p. sodium pentobarbital (MTC Pharmaceuticals, Cambridge, ON) before being sacrificed. Tumor biopsies were either collected and fixed overnight at 4°C in 4% paraformaldehyde/0.1 M PBS pH 7.4, or transferred into 20% sucrose/PBS for another 24 h and then frozen at −80°C until use. Hematoxylin and eosin staining or immunofluorescence were performed on 5-μm thick sections. Green fluorescent BMSC that had engrafted within the tumor were visualized by fluorescent microscopy. To visualize CD31 distribution in induced tumors, sections were blocked for 30 min in 10% normal rabbit serum/10% bovine serum albumin/0.5% Triton X-100 in PBS, then incubated for 1 h at room temperature with a primary rat monoclonal antibody (Pharmingen, San Diego, CA) directed against CD31. Sections were rinsed several times in PBS, following which a rhodamine-conjugated rabbit anti-rat IgG antibody (Jackson Laboratories, Bar Harbor, MA) diluted at 1/200 was applied. Sections were again rinsed in PBS and a coverslip was applied over with Vectashield (Vector Laboratories, Burlingame, CA). Total CD31-stained BV were evaluated from five cross sections derived from three independent tumor biopsies, and monitored in three representative microscopic fields. All sections were examined with an Olympus BX60 microscope. Digital images were analyzed with Image Pro software (Media Cybernetics, Carlsbad, CA). In sections stained with anti-

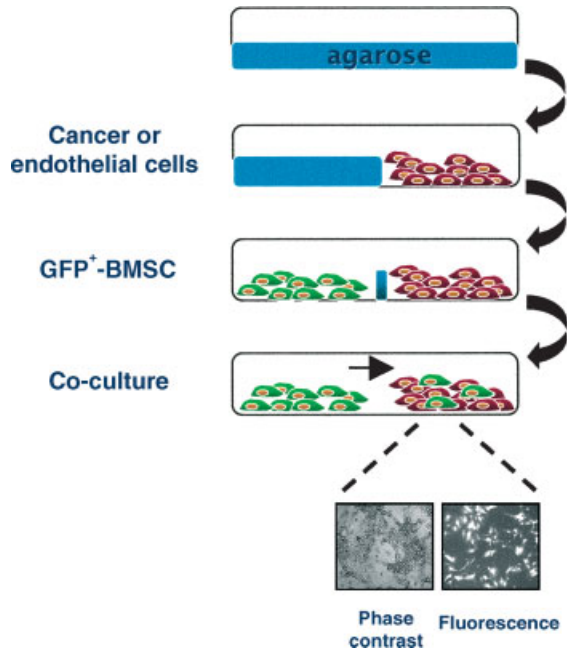

Fig. 1. Scheme of the two-dimensional in vitro angiogenesis assay. Cancerous or endothelial cells are seeded on the right side of the cell culture plate while green fluorescent protein–bone marrow-derived stromal cells (GFP^+–BMSC) are seeded on the left side of that same dish. Visualization of the fluorescent BMSC cells that had migrated from left to right toward the co-cultured cell line is performed several days after the small agarose barrier that separated both cell populations was removed. A micrograph of typical phase contrast (showing both U-87 and BMSC) and fluorescence (only showing GFP^+–BMSC) visualization is shown. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com].

CD31 antibody/peroxidase, we considered as BV only tubular structures within the tumor biopsy that were CD31-positive (i.e., peroxidase positive). For vascular density measurements, we measured the surface area of each section using ×400 magnification and Image Pro software. We considered as BV only tubular structures with potent lumen that were CD31-positive. For vascular density measurements using sections stained with anti-CD31 antibody, we counted the number of BV in ten random high-power fields (hpf) elected using the systemic sampling with random start technique. We calculated the average of the 10 hpf and expressed the vascular density as BV/hpf.

Statistical Data Analysis

Data are representative of three or more independent experiments. Statistical significance was assessed using Student's unpaired *t*-test and was used to compare migration and extent of capillary-like structure formation to untreated (control) basal migration or spontaneous BMSC tube-formation. Probability values of less than 0.05 were considered significant, and an asterisk (*) identifies such significance in each figure.

RESULTS

BMSC Contribute to the Formation of Highly Vascularized Tumor In Vivo

Recent studies, in which BMSC-embedded Matrigel plugs were implanted s.c., provided crucial insight towards the in vivo differentiation of BMSC into CD31-positive EC [Eliopoulos et al., 2002; Al-Khaldi et al., 2003a]. Accordingly, we evaluated whether s.c. co-injection of highly vascularized tumor-derived U-87 glioblastoma cells together with stably transduced GFP^+–BMSC could contribute to enhanced tumor neo-vascularization. As shown in Figure 2A, s.c. injection of 10^6 U-87 glioma cells alone into nude mice induced the formation of a tumor mass within a period of 3 weeks which size and weight were significantly increased when GFP^+–BMSC were co-injected with U-87 cells (U-87 tumors: 1.4 ± 0.7 g, U-87 with BMSC tumors: 4.1 ± 1.7 g). Moreover, co-injection of GFP^+–BMSC with U-87 glioma cells at a 1:1 cell ratio resulted in the formation of a significantly more vascularized tumor over the same period of time (U-87 tumors: 6.1 ± 2.5 vessels/hpf, U-87 with BMSC tumors: 33 ± 8 vessels/hpf) (Fig. 2A). Haematoxylin and eosin-stained sections of the biopsies revealed the presence of basal endogenous tumor vascularization (Fig. 2B) which contrasted strongly with the enhanced size and BV content of tumors that were induced by co-injected BMSC and U-87 cells (Fig. 2B). Tissue sections further show that GFP^+–BMSC co-localized with the basement membrane of several BV (Fig. 2C,D), where some of these GFP-positive cells also stain positively for the EC surface marker CD31 (Fig. 2D). Interestingly, these GFP/CD31-positive structures only represented approximately 5% of the total CD31 stained BV in accordance with previous reports [Al-Khaldi et al., 2003a]. This not only suggests that most of the increased vascularization was attributable to BMSC-mediated paracrine induction and/or recruitment of host-derived BV, but also that BMSC contributed, to some extent, to tumor neo-vascularization.

Spontaneous BMSCs' Capillary-Like Structure Formation on Matrigel Basement Membrane Model Is Dependent on bFGF

Tube formation and sprouting of new capillary vessels represent key events in tumor angiogenesis [Griffioen and Molema, 2000]. BMSC vascular tube formation ability was thus assessed using a Matrigel basement membrane model. This reconstituted basement membrane is known to contain several growth factors such as transforming growth factor β, epidermal growth factor, insulin-like growth factor 1, platelet-derived growth factor, bFGF, and high levels of soluble MMPs, all of which influence cellular network formation [Vukicevic et al., 1992]. Soluble MMPs and VEGF were used as markers for the depletion from Matrigel of its soluble matter. Complete depletion of soluble proMMP-2 and VEGF is shown in Figure 3A, as demonstrated by a highly sensitive gelatin-zymography assay, and it is likely that most of the other Matrigel-derived growth factors were also depleted.

In order to assess the angiogenic properties of BMSC, spontaneous capillary-like network formation was monitored in parallel between BMSC and HMEC, a differentiated human microvascular EC line. Tube formation was induced when BMSC were plated on complete Matrigel and this was similarly observed with HMEC (Fig. 3B). This observation suggests that BMSC share some common endothelial-like

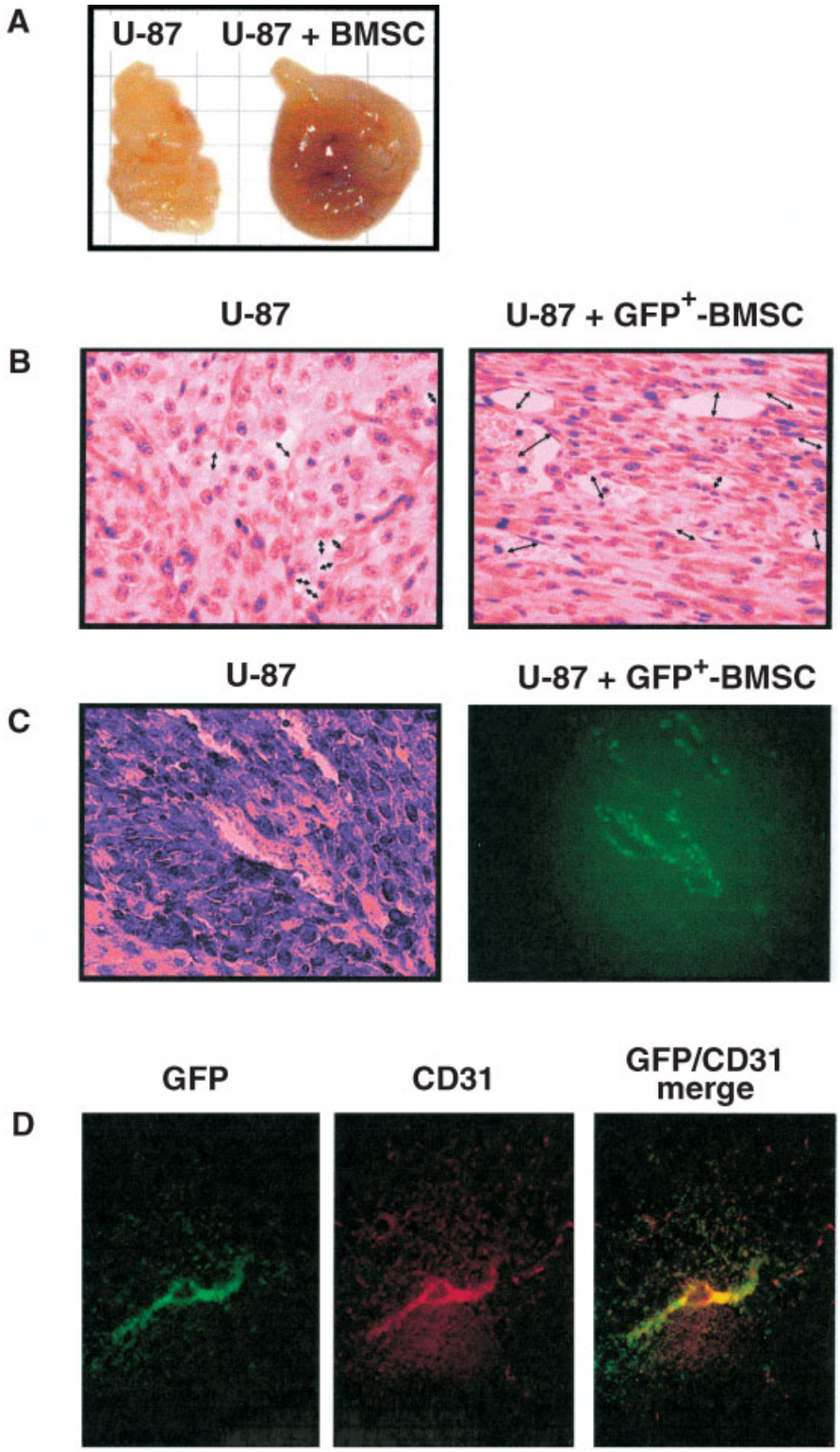

Fig. 2. BMSC contribute to increased tumor neovascularization in vivo. U-87 glioma cells (10^6), alone or in combination with GFP^+–BMSC (10^6), were injected subcutaneously into nude mice and tumors allowed to develop for 3 weeks. **A**: Characteristic tumor biopsies from U-87 or U-87/BMSC. Note the increased neovascularization induced by the presence of BMSC as shown by the formation of blood vessels (BV). **B**: Hematoxylin and eosin staining of paraffin sections of the U-87 and U-87/BMSC tumors. Note the increase in BV number and lumen size characterizing the U-87/BMSC tumors (arrows). **C**: Paraffin sections were visualized under normal or green fluorescent light. Note that GFP^+–BMSC co-localize with the basement membrane of BV, and that (**D**) immunohistochemical staining for CD31 (red) also co-localized with GFP^+–BMSC (green) resulting in yellow staining when both pictures were merged.

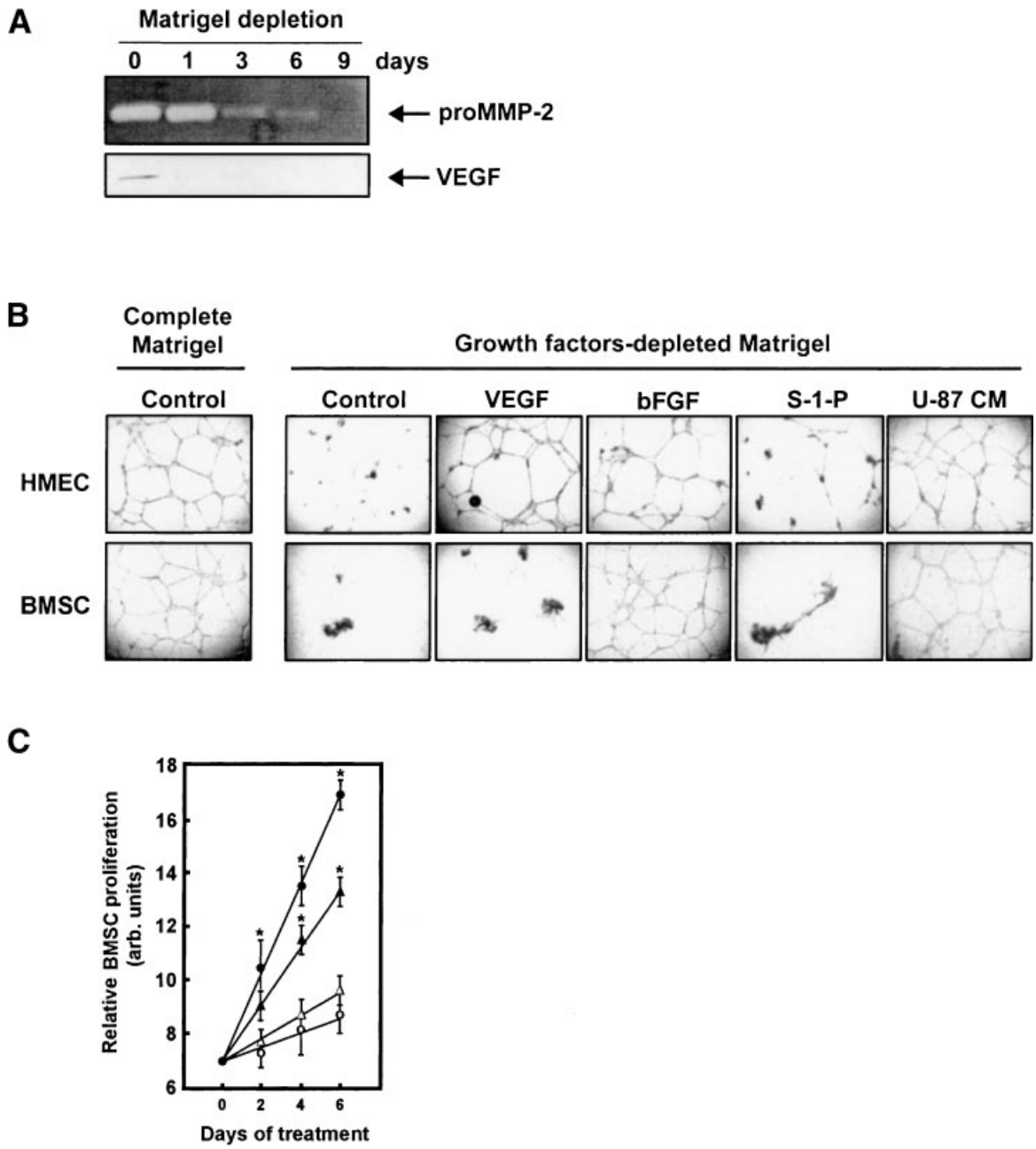

Fig. 3. BMSC proliferation and capillary-like structure formation on a three-dimensional Matrigel basement membrane model is dependent on basic fibroblast growth factor (bFGF). BMSC and human microvascular endothelial cells (HMEC) were seeded either on complete or growth factors-depleted Matrigel and incubated for 18 h at 37°C as described in Materials and Methods. **A**: Gelatin zymogram showing Matrigel depletion of endogenous promatrix metalloproteinase-2 (proMMP-2). **B**: BMSC seeded on depleted Matrigel were supplemented where indicated with 1 ng/ml bFGF, 10 ng/ml vascular endothelial growth factor (VEGF), 1 μM sphingosine-1-phosphate (S1P), or serum-free conditioned media (U-87 CM) isolated from U-87 glioblastoma cells. **C**: BMSC were cultured for the indicated time in the presence of either serum-free media (open circles), 10 ng/ml VEGF (open triangles), 1 ng/ml bFGF (closed circles), or 1 μM S1P (closed triangles) and cell proliferation rates were measured.

phenotype with differentiated EC. In order to further define the role of angiogenic growth factors on the formation of these capillary-like structures, we depleted the Matrigel of most of its endogenous soluble growth factors and protease content as described in Materials and Methods. BMSC and HMEC were then seeded on depleted Matrigel and tube formation was monitored. Interestingly, the ability of both cell types to form capillary-like structures was

lost in Matrigel which had been depleted of growth factors, and only cell aggregates was observed. However, BMSC structure formation was re-induced by the presence of bFGF, but not by VEGF or by S1P, a platelet-derived mediator that we have shown to up-regulate BMSC migration at concentrations as low as 0.1 μM [Annabi et al., 2003b] (Fig. 3B). The VEGF result was not surprising as we have shown that very low levels of VEGF receptors were expressed at the cell surface of BMSC [Eliopoulos et al., 2002]. The lack of any effect of VEGF is further strengthened by the fact that BMSC cultured in the presence of bFGF, but not VEGF, experience a significant increase in their in vitro proliferation rate (Fig. 3C). Interestingly, although S1P concentrations (ranging from 0.1 to 1 μM) known to induce BMSC migration were unable to re-induce BMSC capillary-like structures on depleted-Matrigel (not shown), it significantly triggered BMSC proliferation (Fig. 3C). This suggests that some crucial platelet-derived growth factors may link the processes of hemostasis and angiogenesis. Growth factors-enriched conditioned media isolated from serum-deprived U-87 glioblastoma cells was found to reinduce the formation of these structures on depleted Matrigel (Fig. 3B), further suggesting that paracrine regulation by cancer cells may result in the induction of tubulogenesis in both BMSC and differentiated endothelial cells. The use of a blocking anti-bFGF antibody, however, failed at antagonizing both the chemotactic and capillary-like structure formation capabilities of BMSC in response to conditioned media isolated from cancer cells (not shown). This observation strongly suggests that the growth factors-enriched media potentially contains (yet unidentified) factors that complement (or can even replace) the effects of bFGF alone.

To further explore this assumption, conditioned media was isolated from several serum-deprived cell lines and subsequently used to assess the ability of their growth factors content to reinduce capillary-like structure formation of BMSC on depleted Matrigel. The BMSC capillary-like structure formation was then quantified by measuring the extent of capillary length by surface unit and is reported in Table I. The results obtained confirm that growth factors secreted by both differentiated EC (BAE, PAE pulmonary aortic endothelial), and many of the cancer cells tested (Panc-1 pancreatic carcinoma, Hep-G2 hepatocarcinoma, HT-1080 fibrosarcoma, Caki-1 renal metastatic, U-87 glioblastoma) strongly re-induced formation of capillary-like networks. Interestingly, capillary-like structure formation was also strongly stimulated by conditioned media isolated from starved BMSC themselves, suggesting autocrine regulation of this angiogenic phenotype.

TABLE I. Relative BMSC Capillary-Like Structure Induction by Conditioned Media Isolated From Different Cell Lines

Origin of conditioned media	Relative length of capillary-like structure formation
DMEM−	None
DMEM+	299 ± 5
COS-7	None
NIH 3T3	None
Panc-1	75 ± 53
Hep-G2	280 ± 10
BAE	303 ± 71
PAE	350 ± 16
HT-1080	386 ± 37
Caki-1	428 ± 18
NCTC	435 ± 19
BMSC	438 ± 30
U-87	440 ± 11

BAE, bovine aortic endothelial; DMEM, Dulbecco's modified Eagle's medium.
BMSC were seeded (20,000 cells/well) on depleted Matrigel and cultured in serum-free media (DMEM−), serum-containing media (DMEM+), or conditioned media isolated from cell lines which were serum-deprived for 24 h. Capillary-like structure formation was monitored and quantified by measuring the total relative length of the capillary network.

BMSC Migration Is Induced Through Paracrine Regulation

We also examined whether the capillary-like structure formation capability of BMSC could, in part, be attributed to paracrine or autocrine induction of their migratory potential. The two main pro-angiogenic peptides, VEGF and bFGF, as well as growth factors-enriched conditioned media isolated from normal or cancerous cell lines cultured at initial equal densities and deprived of serum for 18 h, were used as chemoattractants for BMSC migration. BMSC were seeded on top of gelatin-coated filters and assayed for migration in the presence or absence of the conditioned media. BMSC migration was increased by growth factors-enriched conditioned media to different extents, depending on the nature of the conditioned media (Fig. 4). Most culture media which had been conditioned by tumor-derived cell lines such as U-87, HT-1080, Caki-1, or Hep-G2 strongly induced (sixfold to eightfold) BMSC migration.

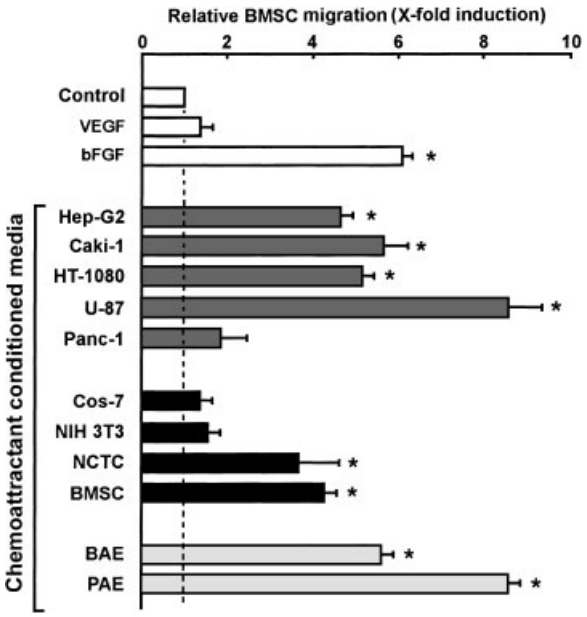

Fig. 4. Secreted angiogenic factors from cancer and endothelial cell lines induce BMSC migration. Conditioned media were isolated from different serum-deprived cell lines, and used as chemoattractant in the lower compartment of modified Boyden chambers. BMSC (10^5) were seeded on top of gelatin-coated filters and allowed to migrate for 2 h. Conditioned media was isolated from equal cell densities of NIH 3T3 normal fibroblastic cells, marrow stromal cells (BMSC), bovine aortic endothelial (BAE) cells, COS-7 epithelial cells, Caki-1 renal metastatic cells, U-87 glioblastoma cells, NCTC murine macrophage cells, pulmonary aortic endothelial (PAE) cells, HT-1080 fibrosarcoma cells, Hep-G2 hepatocarcinoma cells, and PANC-1 pancreatic carcinoma cells.

However, Panc-1 conditioned media only induced a marginal increase in BMSC migration. Conditioned media from EC lines such as BAE and PAE also caused increases in BMSC migration that were similar to those observed with media from the most potent cancer cell lines tested. It is thus tempting to suggest that growth factors secreted from both cancer and EC may regulate BMSC migratory potential and may involve members of the FGF family. Indeed, bFGF alone induced BMSC migration by sixfold, while VEGF only had a marginal effect on cell migration (Fig. 4). Conditioned media isolated from normal epithelial COS-7 or fibroblastic NIH 3T3 cell lines did not induce a significant increase in BMSC migration, while media isolated from NCTC macrophage cells significantly induced BMSC migration. Consistent with its ability to re-induce capillary-like structure formation, some autocrine regulation of BMSC migratory potential is also suggested, since conditioned media of serum-deprived BMSC contained growth factors that also induced BMSC migration.

BMSC Recruitment Is Triggered by Endothelial and Cancer Cell Co-Culture

Since paracrine regulation of BMSC migration and capillary-like structure formation on Matrigel can be attributed, in part, to secreted exogenous growth factors, we further examined the regulation of these cell properties using a two-dimensional co-culture assay consisting of BMSC being co-cultured alongside endothelial or cancer cells (see scheme in Fig. 1). We thus plated, on one half of the culture flask, BMSC which had been engineered to stably express GFP, and either non-EC (COS-7, NIH-3T3), EC (BAE, HMEC), or cancer cell lines (U-87, HT-1080, MCF-7, Caki-1) on the other half of the culture flask. Interestingly, BMSC were strongly attracted to both cancer and EC, as indicated by directional migration over a period of 96 h (Fig. 5). No significant recruitment was observed when BMSC were co-cultured in the presence of epithelial (COS-7) or fibroblast (NIH 3T3) cells. This cell–cell co-culture approach clearly demonstrates that factors secreted from cancer cells or EC lines induce a selective recruitment and interaction with BMSC, and supports the observed in vivo induction of tumor vascularization (Fig. 2).

DISCUSSION

Molecular studies of the phenotypical and endothelial-like functional properties of BMSC in neo-vascularization, and their role in microvascular network remodeling in response to tumor angiogenic factors, have received little attention. The recently reported unorthodox plasticity and phenotype of BMSC [Huss et al., 2000; Tomanek and Schatteman, 2000; Al-Khaldi et al., 2003a] thus provides new insights into their potential role in tumor vascularization. This is further strengthened by the role that bone marrow-derived endothelial progenitor cells already play in promoting neo-angiogenesis [Asahara et al., 1997, 1999]. The present study revolves around the working hypothesis that a crucial role of BMSC in support of tumor neo-vascularization may, in part, be attributable to paracrine regulation of their angiogenic properties. Indeed, we provide evidence that (i) BMSC can participate in active tumor angiogenic processes as they are massively

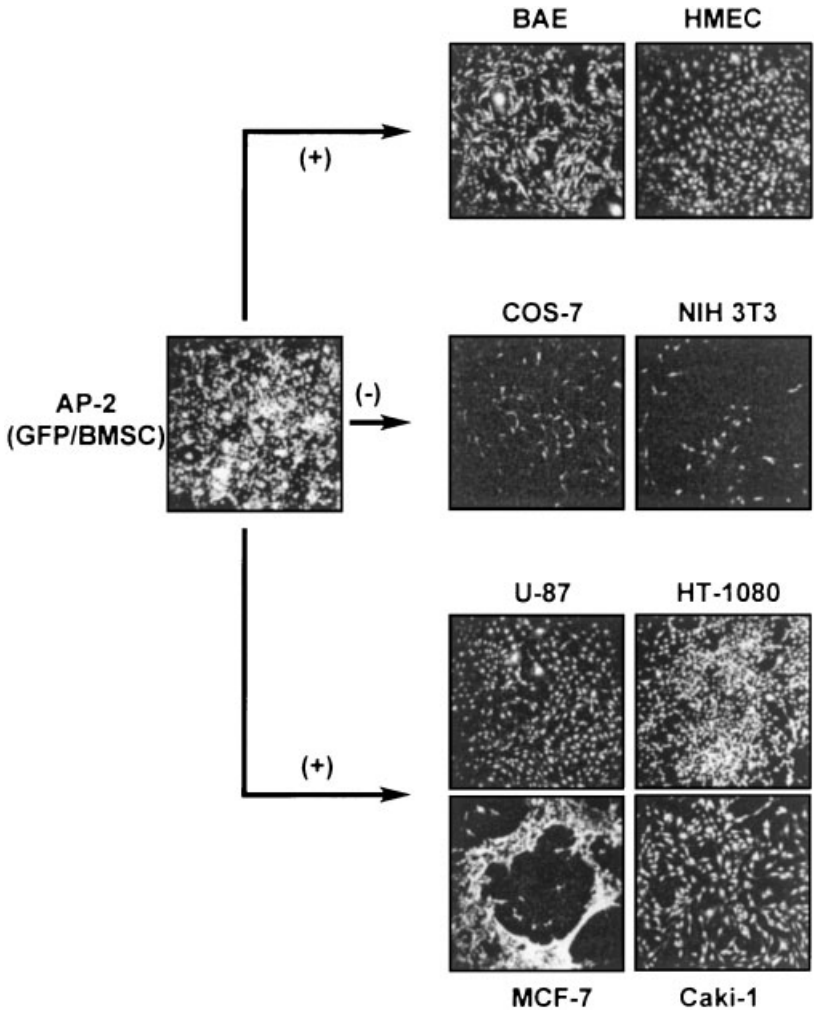

Fig. 5. Cancer and endothelial cell-dependent paracrine regulation of BMSC recruitment and morphological changes. Stably GFP-transfected BMSC (extreme **left panel**) were co-cultured on the left half of a 6-well cell culture plate. The right sides of the wells contained either a cancer cell line (U-87 glioma cells, MCF-7 breast cancer cells, HT-1080 fibrosarcoma cells, or Caki-1 metastatic kidney cells), or a non-cancerous cell line (COS-7 epithelial kidney cells, NIH 3T3 fibroblast cells, or BAE cells). Co-cultures were performed at 37°C for 4 days. Recruitment and morphological changes of GFP^+–BMSC were monitored using fluorescent microscopy; (+) indicates that BMSC were recruited and migrated towards the indicated cell lines, while (–) indicates very low recruitment of BMSC.

recruited by both differentiated EC and cancer cells; (ii) growth factors-mediated paracrine regulation promotes expression of a bFGF-dependent angiogenic phenotype in BMSC including strong migratory behavior and the ability to generate capillary-like structures similar to those formed by differentiated EC; (iii) BMSC adopted immunophenotypic characteristics consistent with EC functionally related to vascular structures; and that (iv) BMSC contributed to autocrine and/or paracrine-enhanced in vivo tumor vascularization. Collectively, these endothelial-like properties of BMSC suggest that they may be involved in angiogenic processes, including cell proliferation, migration, tube formation, and vessel stabilization. All of these processes could also be regulated at an early stage of BMSC differentiation by pro-angiogenic peptides involving members of the FGF family [Westphal et al., 2000; Ribatti et al., 2001], which have been reported to be abundantly produced in a tumor origin-specific manner as well as by human glioma tissues [Gospodarowicz, 1989; Takahashi et al., 1991].

In light of these recent studies, BMSC may thus share with differentiated EC some common molecular and cellular phenotypes, with regard to the tumor microenvironment, that may actively regulate their involvement in

angiogenesis. One such common cellular phenotype between BMSC and differentiated EC is BMSC's ability to generate membrane-type-1 MMP-dependent capillary-like structures [Annabi et al., 2003a], which was also shown to be effective in macro- and micro-vascular EC tubulogenesis [Lafleur et al., 2002]. Moreover, as part of our analytical approach in the present study, we have observed that most of the BMSC-induced in vivo neovascular response was host-derived (Fig. 2). This phenomenon was already reported by us [Al-Khaldi et al., 2003a] and by others who used bone marrow-derived cells and have observed that the tumor-induced neovascularization was only in part derived from bone marrow precursors [Davidoff et al., 2001]. This reflects the extraordinarily potent recruitment effect due to BMSC, beyond their direct contribution to new vessel formation via transdifferentiation. In accordance with this observation, we have recently demonstrated that de novo production of VEGF by BMSC was mediated by hypoxic culture conditions and that migration and tube formation were induced by experimental hypoxic conditions mimicking those found within tumor masses [Annabi et al., 2003a]. Since the *VEGF* gene is hypoxia responsive, it is thus tempting to suggest that reduced oxygen tension, such as that encountered within tumor masses, may provide the stimulus required to locally promote VEGF production by BMSC and to simultaneously initiate a potent host-derived angiogenic response.

One limitation of our study that needs to be admitted is the ability to isolate absolutely pure initial populations of BMSC devoided from virtually any endothelial precursor, smooth muscle, or fibroblast contaminants. Further phenotypic characterization of our BMSC subpopulation will help define whether the vascular progenitors derived from BMSC express endothelial markers such as VEGFR-2 or VEGF-3 upon cancer cell recruitment or Matrigel-induced differentiation. Such phenomenon was indeed observed in subsets of circulating human $CD133^{+}CD34^{+}$ stem and progenitor cells derived from bone marrow and which could potentially contribute to neo-angiogenesis in wound healing and tumor growth [Peichev et al., 2000; Salven et al., 2003].

BMSC were recently shown to exhibit cardiomyogenic phenotypes that enable them to replace native cardiomyocytes when implanted into myocardium [Wang et al., 2000]. Moreover, we have also shown that BMSC differentiated into vascular smooth muscle (positive α-smooth muscle actin), skeletal muscle (positive desmin), and adipocytes in an ischemic hind limbs where BMSC were implanted [Al-Khaldi et al., 2003b]. BMSC are thus capable of spontaneously regenerating the various components of muscular tissues. This demonstrates that BMSC are indeed multipotent with respect to the microenvironment in which they engraft. Accordingly, genetically engineered BMSC can thus be used in different therapeutic strategies as they are not rejected and are known to escape the immune system. BMSC have indeed been shown to exert immunoregulatory activity potential and to physically inhibit T-cells [Krampera et al., 2003]. Baboon BMSC have also been observed to alter lymphocyte proliferation and reactivity to allogeneic target cells and tissues [Bartholomew et al., 2002; Tse et al., 2003]. This issue is a crucial one with respect to the eventual use of BMSC in cancer treatment since the immuno-suppressive properties of BMSC at the moment of their injection may not only result in decreased immune control of the tumor but ultimately limit their therapeutical use in cancer treatment. The sum of these published data clearly urges one to explore these potential BMSC side-effects as these cells are already in use as vehicle in gene therapy strategies.

In conclusion, we have shown that BMSC have the ability to be recruited at active sites of angiogenesis and to promote a robust host-derived angiogenic response in vivo. These observations are consistent with a recent study suggesting that BMSC also participate in angiogenesis and arteriogenesis de novo [Al-Khaldi et al., 2003a]. While therapeutic revascularization associated with transplanted BMSC may in part result from host-derived angiogenic responses, our observations imply that the molecular and cellular phenotypes of BMSC may also ultimately promote tumor angiogenic processes through BMSC transdifferentiation. Alternatively, recent analysis of gene expression profile of BMSC, as determined by cDNA microarray analysis, revealed a close association of BMSC with vascular cells and indicated that BMSC resemble pericytes [Wieczorek et al., 2003]. Stabilization of vascular barrier integrity may thus be another potentially important role of BMSC and raises the possibility that this may facilitate BV

formation and vascular remodeling at sites of transplanted stem cells.

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