

Endothelial Differentiation of Hematopoietic Stem and Progenitor Cells from Patients with Multiple Myeloma

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Abstract Purpose: Vasculogenesis is a physiologic process typical of fetal development in which new blood vessels develop from undifferentiated precursors (or angioblasts). In tumors, near angiogenesis, vasculogenesis contributes to the formation of the microvascular plexus that is important for diffusion. Here, we show that hematopoietic stem and progenitor cells (HSPC) of multiple myeloma (MM) patients are able to differentiate into cells with endothelial phenotype on exposure to angiogenic cytokines.

Experimental Design: Circulating HSPCs were purified with an anti-CD133 antibody from patients with newly diagnosed MM before autologous transplantation and exposed to vascular endothelial growth factor (VEGF), fibroblast growth factor-2 and insulin-like growth factor in a 3-week culture.

Results: HSPCs gradually lost CD133 expression and acquired VEGF receptor-2, factor VIII – related antigen, and vascular endothelial-cadherin expression. The expression pattern overlapped with paired MM endothelial cells (MMEC). During culture, cells adhered to fibronectin, spread, and acquired an endothelial cell shape. Differentiated HSPCs also became capillarogenic in the Matrigel assay with maximal activity at the third week of culture. Bone marrow biopsies revealed HSPCs inside the neovessel wall in patients with MM but not in those with monoclonal gammopathy of undetermined significance.

Conclusions: In patients with MM, but not in those with monoclonal gammopathy of undetermined significance, HSPCs contribute to the neovessel wall building together with MMECs. Therefore, besides angiogenesis, HSPC-linked vasculogenesis contributes to neovascularization in MM patients. Tentatively, we hypothesize that in HSPC cultures a multipotent cell population expressing low VEGF receptor-2 levels corresponds to the endothelial progenitor cell precursor and seems to be the MMEC precursor.

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Received 8/31/07; revised 12/5/07; accepted 12/19/07.

Grant support: Associazione Italiana per la Ricerca sul Cancro (National and Regional Funds), Milan, Italy; Ministry of Universities and Research (PRIN Projects 2005 and CARSO Project n. 72/2); and Ministry of Health, Progetto Oncologia 2006, Humanitas Mirasole S.P.A., Rome, Italy. T. Cirulli is a recipient of a fellowship from Fondazione Italiana per la Ricerca sul Cancro, Milan, Italy.

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doi:10.1158/1078-0432.CCR-07-4071

During embryo life, blood vessels first appear as the result of vasculogenesis (i.e., the formation of capillaries from endothelial cells differentiating *in situ* from groups of mesodermal cells). The primitive heart and primitive vascular plexus form in this way (1). Remodeling of the primary vascular plexus into a more mature vascular system is thought to occur by angiogenesis, a term applied to the formation of capillaries from existing vessels [i.e., capillaries and postcapillary venules (2)], based on endothelial sprouting or intussusceptive (nonsprouting) microvascular growth (2).

Extensive data support the existence of endothelial progenitor cells (EPC), their bone marrow origin, and contribution to the formation of new blood vessels in adult (3). Their discovery led to the new concept that vasculogenesis and angiogenesis may occur simultaneously in the postnatal life because EPCs are able to differentiate through a mechanism recapitulating embryo vasculogenesis. EPCs are capable to proliferate, migrate, and differentiate into endothelial lineage cells but have not yet acquired characteristics of mature endothelial cells. EPCs mobilized from bone marrow into the blood stream may be recruited and incorporated into sites of active neovascularization during tumor growth (3).

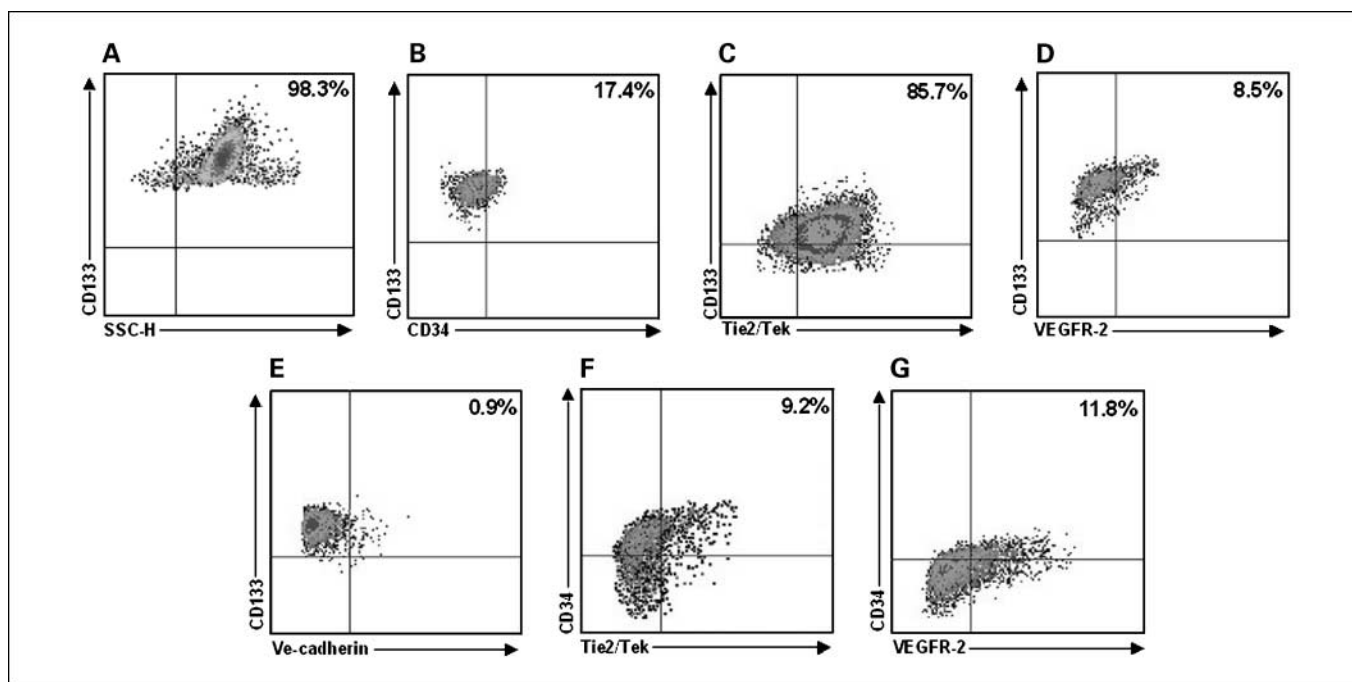


Fig. 1. FACS analysis of undifferentiated HSPCs in a representative patient. At least 10,000 cells were read for each labeling.

The majority of circulating EPCs reside in the bone marrow in close association with hematopoietic stem and progenitor cells (HSPC) and the bone marrow stroma that provides an optimal microenvironment. Yin et al. (4) firstly showed that HSPCs are the CD133⁺ cell population in the bone marrow and peripheral and cord blood. Peichev et al. (5) showed that a small subset of CD34⁺ HSPCs expresses both CD133 and vascular endothelial growth factor (VEGF) receptor-2 (VEGFR-2/KDR) and that incubation of this subset with VEGF, fibroblast growth factor-2 (FGF-2), and collagen results in their proliferation and differentiation into CD133⁺, VEGFR-2⁺ mature endothelial cells.

The importance of angiogenesis for the growth and expansion of solid and hematologic tumors has been amply shown. In multiple myeloma (MM), bone marrow angiogenesis measured as microvascular density increases with progression from monoclonal gammopathy of undetermined significance (MGUS) to MM and is related with the plasma cell labeling index (6). Reciprocal positive and negative interactions between plasma cells and bone marrow stromal cells, including HSPCs, fibroblasts, osteoblasts/osteoclasts, chondroclasts, endothelial cells, EPCs, T cells, macrophages, and mast cells, mediated by an array of cytokines, receptors, and adhesion molecules, modulate the angiogenic response in MM (7). It has been suggested that the presence of EPCs in patients with MM correlates with clinical outcome (8).

Data to be presented show *in vitro* generation of mature endothelial cells from HSPCs of MM patients and provide evidence that HSPCs contribute to bone marrow neovascularization through vasculogenesis.

Materials and Methods

Cell purification and cultures. CD133⁺ HSPCs were harvested from peripheral blood of 25 newly diagnosed MM patients before autologous

stem cell transplantation, after obtaining informed consent in accordance with the Declaration of Helsinki and guidelines of the Local Ethics Committee at the University of Bari Medical School (Bari, Italy). HSPCs were mobilized with cyclophosphamide and granulocyte colony-stimulating factor (9). Leukapheresis was done using a COBE Spectra cell separator (Gambro, Inc.). A CliniMACS device (Miltenyi Biotec) selected CD133⁺ cells by means of anti-CD133 monoclonal antibody-coated magnetic beads. Cell purity ($\geq 98\%$) was assessed with fluorescence-activated cell sorting (FACS) analysis (FACSscan, Becton Dickinson). Then, 1×10^6 CD133⁺ HSPCs per patient were cultured in 75 cm² flasks in complete medium (Iscove's modified Dulbecco's medium, supplemented with 10% fetal bovine serum, 1% glutamine, 100 units penicillin, and 1,000 units streptomycin) and replaced every 4 to 5 d.

MM endothelial cells (MMEC) were harvested from bone marrow of the 25 MM and 18 MGUS patients as described by Vacca et al. (10) and cultured in RPMI 1640 supplemented with 10% FCS and 1% glutamine.

HSPC phenotype and differentiation. The HSPC phenotype was assessed by FACS with anti-CD133, anti-CD34 (a stem cell and endothelial cell marker), anti-tyrosine kinase with Ig and epidermal growth factor homology-2 (Tie2/Tek, a stem cell and endothelial cell marker), anti-VEGFR-2 (an endothelial cell marker), and anti-vascular endothelial (VE)-cadherin (an endothelial cell marker) monoclonal antibodies, all from Immunotech.

To induce endothelial cell differentiation, HSPCs were replated at 1×10^5 to 2×10^5 cells/cm² in fibronectin-coated plates or chamber slides in complete medium supplemented with 10% horse serum, 10^{-8} mol/L dexamethasone, 50 ng/mL VEGF, 10 ng/mL FGF-2, and 10 ng/mL insulin-like growth factor (IGF), all from Calbiochem, which are the most important angiogenic cytokines secreted by bone marrow plasma cells (6), and changed every 4 to 5 d. In some instances, cells were subcultured after ~ 9 d at a 1:4 dilution under the same culture conditions for a maximum of 20 population doublings.

Reverse transcription-PCR. Total RNA (2 μ g) was extracted with Trizol reagent (Invitrogen, Life Technologies) and reverse transcribed by Moloney murine leukemia virus reverse transcriptase (Invitrogen). Then, 1 μ g cDNA was amplified by 35 cycles with primers

for human CD133 (5'-TACCAAGGACAAGGCGTTCAC-3' and 5'-CAGTCGTGGTTTGGCGTTGTA-3') and human factor VIII-related antigen (FVIII-RA; an endothelial cell marker: 5'-GTTCGTCTGGAAGGATCGG-3' and 5'-CACTGACACCTGAGTGAGAC-3'), by 30 cycles with primers for human VEGFR-2 (5'-CTTACCCAGGATATGGAG-3' and 5'-CCGTCAAGGGAAAGACTACG-3') and human VE-cadherin (5'-CCCGTCTTTACTCAATCCACA-3' and 5'-GGGTTTGATGATACCCTCGTT-3'), and by an equal number of cycles per test with primers for the control glyceraldehyde-3-phosphate dehydrogenase (5'-CCCTCCAAAATCAAGTGGGG-3' and 5'-CGCCACAGTTTCCCGGAGGG-3'). Band intensity was expressed as arbitrary absorbance units by reading with Fluorad (Bio-Rad Laboratories) and the Kodak 1D 3.5 image analysis system (Eastman Kodak Co.), which converts the band density into number of pixels.

Western blot. Proteins (50 µg) were subjected to 8% SDS-PAGE under reducing conditions, electrotransferred to a polyvinylidene difluoride membrane (NEN Life Science), and incubated with rabbit antiserum against FVIII-RA (Dako), the same monoclonal antibodies against CD133, VEGFR-2, and VE-cadherin used on FACS, and the anti-VEGFR-1 monoclonal antibody (Immunotech), and then with

horseradish peroxidase-conjugated secondary antibodies. Enhanced chemiluminescence (NEN Life Science) was revealed by Kodak BioMax film (Eastman Kodak). Band intensity was expressed as arbitrary absorbance, as described for reverse transcription-PCR.

Immunofluorescence microscopy. HSPCs cultured on fibronectin-coated chamber slides were fixed on days 0, 7, 14, and 21 with 4% paraformaldehyde, permeabilized in 0.2% Triton X-100, blocked with 3% bovine serum albumin in PBS, incubated for 1 h with the same anti-CD133, anti-VEGFR-2, and anti-VE-cadherin primary antibodies used on FACS and anti-FVIII-RA antibody used on Western blot, and then incubated for 45 min with the corresponding FITC-labeled goat anti-mouse IgG or goat anti-rabbit IgG secondary antibodies (all from Sigma-Aldrich). Slides were observed on a Zeiss Axioplan 2 fluorescence microscope (Zeiss).

Capillarogenesis on Matrigel. This *in vitro* assay assessed differentiated HSPC production of neovessels in three-dimensional vascular tubes and cord-like structures connecting "cellular nodes," which resemble an organized capillary mesh. On days 0, 11, and 21 of culture, HSPCs were plated in duplicate in 24-well plates (2×10^5 cells per well) precoated with Matrigel (300 µL/well; Becton Dickinson) in 1 mL/well of serum-free medium admixed with 50 ng/mL VEGF plus 10 ng/mL FGF-2. After

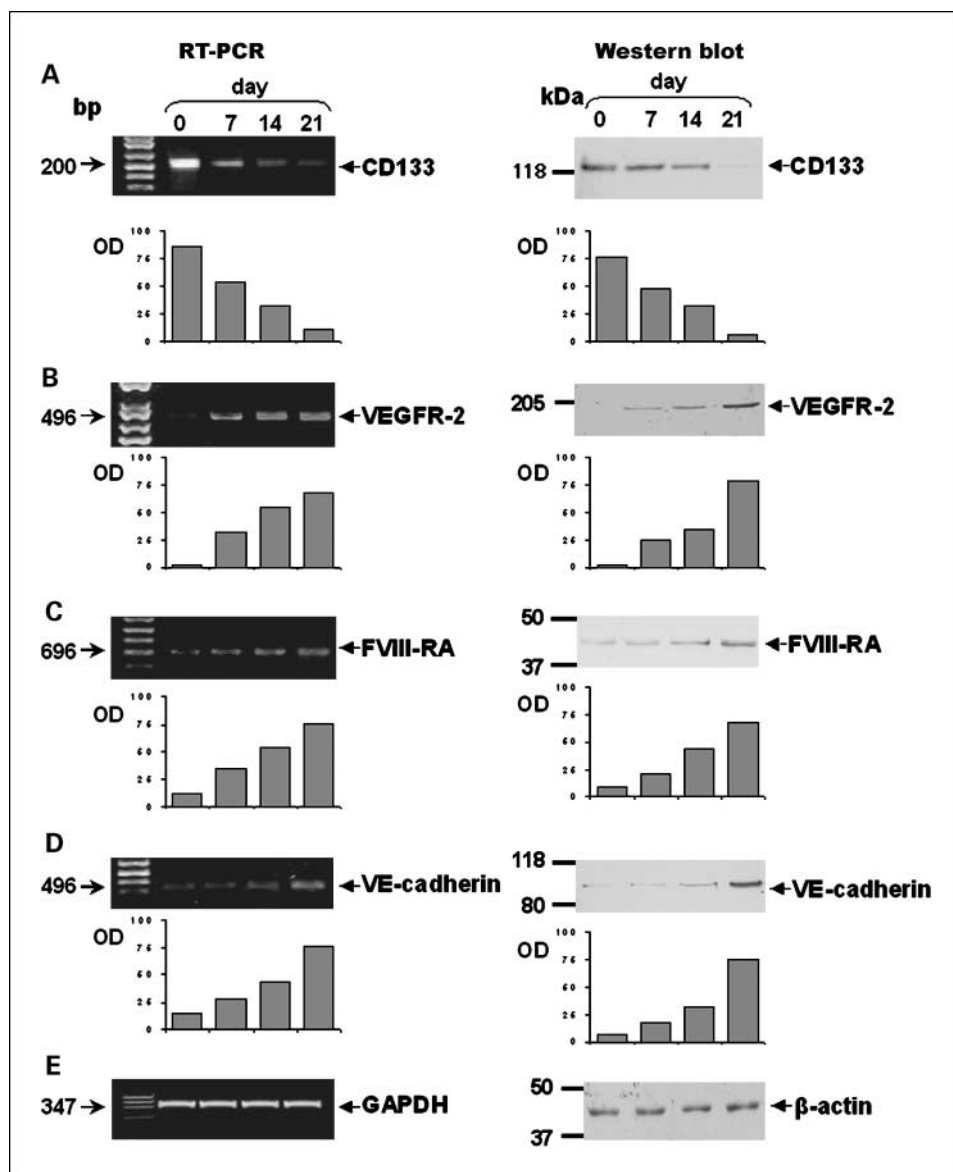
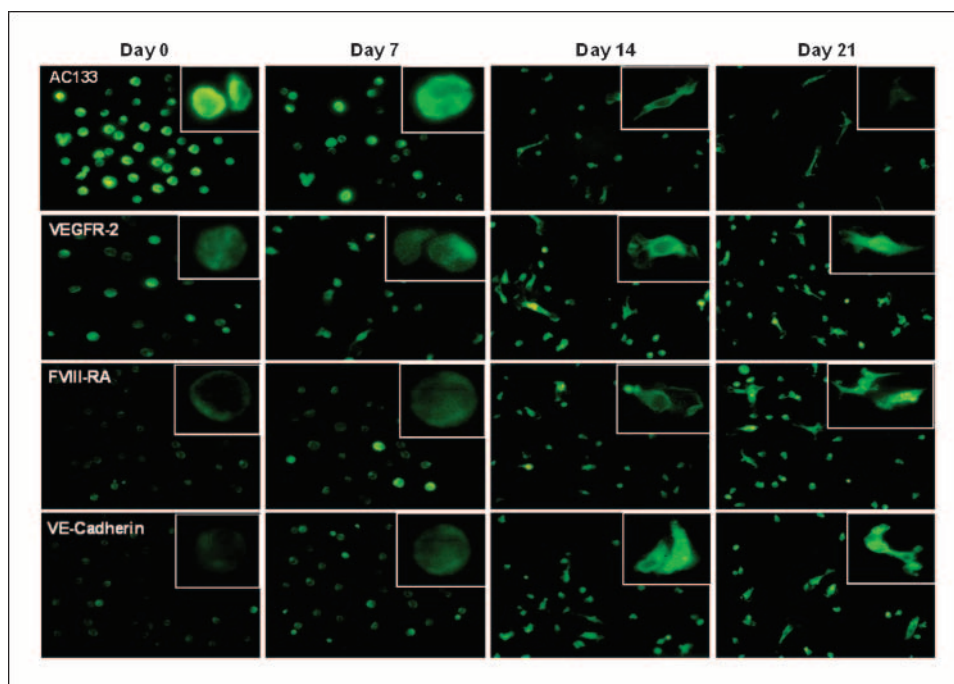


Fig. 2. mRNA: expression of CD133 and endothelial cell markers and protein transcription during HSPC differentiation. Histograms show densitometric analysis of bands as absorbance (OD) in a representative patient. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β -actin were used as controls. bp, base pairs.

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Fig. 3. Immunofluorescence analysis of CD133 and endothelial cell markers during HSPCs differentiation in a representative patient. Note the gradual loss of CD133 and the parallel increase of VEGFR-2, FVIII-RA, and VE-cadherin on HSPCs during their differentiation culture. Insets, changes in HSPC shape.



12-h incubation, the three-dimensional organization was examined under a reverted, phase-contrast photomicroscope. Capillary mesh topological variables [empty mesh area count, vessel length (μm), and mesh branching point count] were measured by computed image analysis, as described by Vacca et al. (11), and expressed as mean \pm 1 SD.

Laser scanning confocal microscopy. Decalcified sections of bone marrow biopsies from MM and MGUS patients were deparaffinized, washed in PBS, and incubated for 1 h with primary anti-CD133, anti-VEGFR-2, anti-VE-cadherin, and anti-Tie2/Tek monoclonal antibodies and rabbit antiserum against FVIII-RA. Sections were washed with PBS/bovine serum albumin and incubated for 1 h with the secondary FITC-conjugated anti-mouse or TRITC-conjugated anti-rabbit antibody. In some experiments, the nuclei were counterstained with 4',6-diamidino-2-phenylindole. Confocal microscopy was done with a Leica TCSSP2 microscope (Leica Microsystems GmbH). Fluorescent signals by FITC-conjugated (excitation, 490 nm; emission, 516 nm) and TRITC-conjugated (excitation, 544 nm; emission, 572 nm) secondary antibodies were quantified by the Leica confocal software (LCS-TCS version 2.61). Using the "stack" function for the defined area, the software produced a xz intensity profile of the average pixel value within marked edges, including a single cell, as a function of each focal plane. Correction was made for the minimal background by repeating the procedure in a cell-free field. The integrated value of the xz profile was taken as a measure of individual cell fluorescence intensity relative to the selected emission channel. About 100 single cells were analyzed for both emission channels.

A "HSPC score" was defined as the percentage of neovessels formed by at least one CD133⁺ HSPC within total FVIII-RA⁺ neovessels in four to six $\times 250$ fields covering each of two sections per biopsy.

Results

HSPC phenotype. More than 98% of HSPCs expressed CD133 on FACS analysis (Fig. 1A). Double staining revealed that $18.3 \pm 3.2\%$ of CD133⁺ HSPCs expressed CD34 (Fig. 1B), $88.6 \pm 8.3\%$ expressed Tie2/Tek (Fig. 1C), $7.6 \pm 1.6\%$ expressed VEGFR-2 (Fig. 1D), and $1.2 \pm 0.8\%$ expressed VE-cadherin (Fig. 1E). HSPCs were double stained with CD34 and Tie2/Tek in $9.5 \pm 0.7\%$ (Fig. 1F) and with CD34 and VEGFR-2

in $13.6 \pm 2.1\%$ (Fig. 1G). The marginal VEGFR-2 and VE-cadherin expression indicated that CD133⁺ HSPCs were not orientated to endothelial cell differentiation. High CD133 expression and marginal VEGFR-2 and VE-cadherin expression were confirmed at mRNA, reverse transcription-PCR, and Western blot (day 0; Fig. 2A, B, and D). FVIII-RA was very little expressed (Fig. 2C), further confirming that CD133⁺ HSPCs were not engaged in the endothelial cell differentiation pathway.

HSPCs differentiate into cells with endothelial features. To induce endothelial cell differentiation, HSPCs were seeded in fibronectin-coated wells in medium supplemented with VEGF, FGF-2, and IGF and analyzed on days 7, 14, and 21 with reverse transcription-PCR, Western blot (Fig. 2), and immunofluorescence (Fig. 3). Figures 2 and 3 show that CD133 was progressively lost, whereas the endothelial cell markers VEGFR-2, FVIII-RA, and VE-cadherin increased in a time-dependent manner, peaking on day 21. In addition, the expression of these endothelial cell markers increased in intensity and percentage of positive cells (Fig. 3). VEGFR-1 behaved similarly to VEGFR-2 (Supplementary Fig. S1).

As HSPCs differentiated toward the endothelial cell phenotype, they gradually acquired the typical endothelial cell shape (Fig. 3, insets). On day 0, cells were small and rounded and most grew in suspension; with exposure to VEGF/FGF-2/IGF, cells progressively adhered to the fibronectin substrate, becoming larger and spindle shaped.

On day 21, HSPC-derived endothelial cells and resting paired MMECs showed an overlapping CD133, VEGFR-2, FVIII-RA, and VE-cadherin expression pattern (Fig. 4).

Capillarogenesis by HSPCs. On exposure to VEGF/FGF-2/IGF, HSPCs acquired the ability to form a capillary-like network in step with differentiation toward endothelial cells. On day 0, HSPCs seeded on Matrigel for 12 h remained spherical in shape and isolated or aggregated in small clumps without forming tubular structures (Fig. 5A and D). On day 11, cells produced a

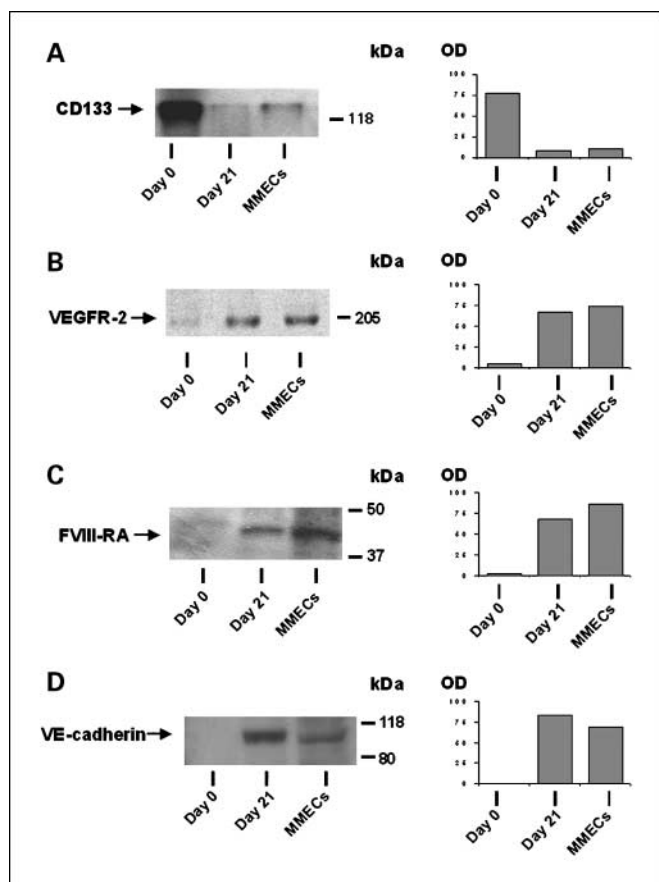


Fig. 4. Expression of CD133 and endothelial cell markers by undifferentiated (day 0) and differentiated (day 21) HSPCs compared with resting paired MMECs in a representative patient. Histograms show densitometric analysis of bands, as described in Fig. 1.

thin, loose mesh with few branching points (Fig. 5B and E). The mean empty mesh area count was 26.2 ± 5.2 , the mean vessel length was $3,940 \pm 650 \mu\text{m}$, and the mean mesh branching point count was 106 ± 24 . On day 21, cells spread through the Matrigel surface and aligned to form numerous branching, anastomosing tubes with multicentric junctions that were arranged in a closely knit capillary-like mesh (Fig. 5C and F).

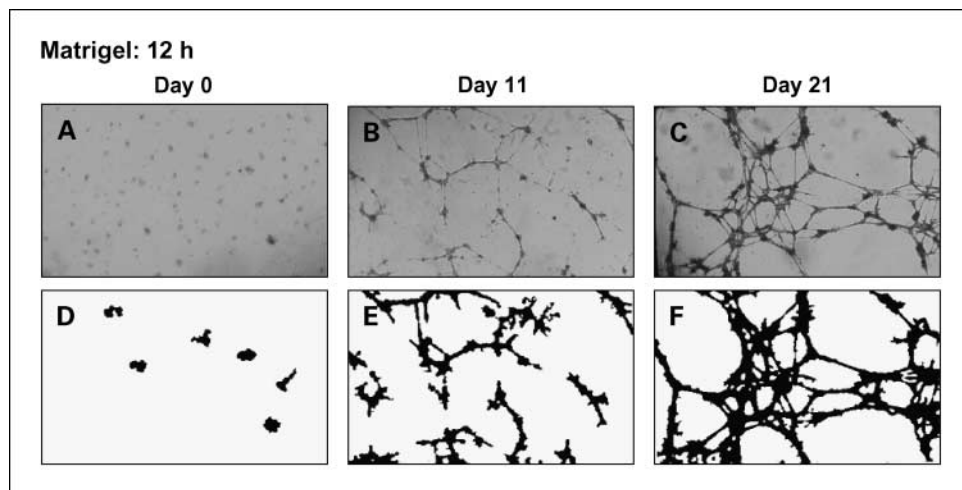


Fig. 5. Capillarogenesis on Matrigel. On the days indicated, HSPCs were seeded on Matrigel in serum-free medium added with VEGF and FGF-2. Note that on day 21 HSPCs form a closely knit vascular network.

The mean empty mesh area count was 62.4 ± 8.3 , the mean vessel length was $7,142 \pm 1,976 \mu\text{m}$, and the mean mesh branching point count was 198 ± 37 ($P < 0.01$, Wilcoxon-Wilcox test).

Confocal microscopy of bone marrow biopsies. Using confocal microscopy of bone marrow biopsies, we observed that HSPCs contributed to neovessel wall assembly in 23 of 25 MM patients and in 1 of 18 patients with MGUS. Figure 6 shows that some FVIII-RA⁺, VEGFR-2⁺, VE-cadherin⁺, and Tie2/Tek⁺ MMECs (green stained in Fig. 6A-D) are scattered in the bone marrow and coexpress CD133 (red stained). CD133⁺ HSPCs were found inside the neovessel wall together with FVIII-RA⁺, VEGFR-2⁺, and VE-cadherin⁺ MMECs (Fig. 6E, F, and G, respectively). CD133⁺ HSPCs may be differentiating into FVIII-RA⁺, VEGFR-2⁺, or VE-cadherin⁺ MMECs. In contrast, figures of this type were absent in MGUS (Fig. 6H), with the exception of only one patient. Thus, the HSPC score was 12 ± 6 in the MM patients and 1 ± 1 in those with MGUS.

Discussion

In the early phases of the development of the vascular system, endothelial cells are derived from undifferentiated mesodermal cells. Endothelial cells and hematopoietic cells come from a common progenitor, the hemangioblast, found in the aorta-gonad-mesonephros region (12, 13) and in fetal liver (14). The ontogenic development of hemangioblast is linked to VEGFR-2 because mice lacking VEGFR-2 display a defect in both hematopoietic cells and endothelial cells (13, 15–17). In human postnatal life, CD133⁺, CD34⁺, and VEGFR-2⁺ cell subsets in bone marrow and peripheral and cord blood are endowed with the functional activity of hemangioblasts because they are able to differentiate in both hematopoietic cells and endothelial cells (18, 19).

In prenatal life, commitment of the hemangioblast to the endothelial lineage is characterized by the sequential expression of VE-cadherin, CD31, and CD34 (20, 21), whereas in postnatal life EPCs have been selected from bone marrow and blood using antibodies to CD133, VEGFR-2, CD34, and MUC18 (or CD146; refs. 5, 22, 23). On differentiation to mature endothelium, CD133, CD34, and MUC18 are lost (5, 22).

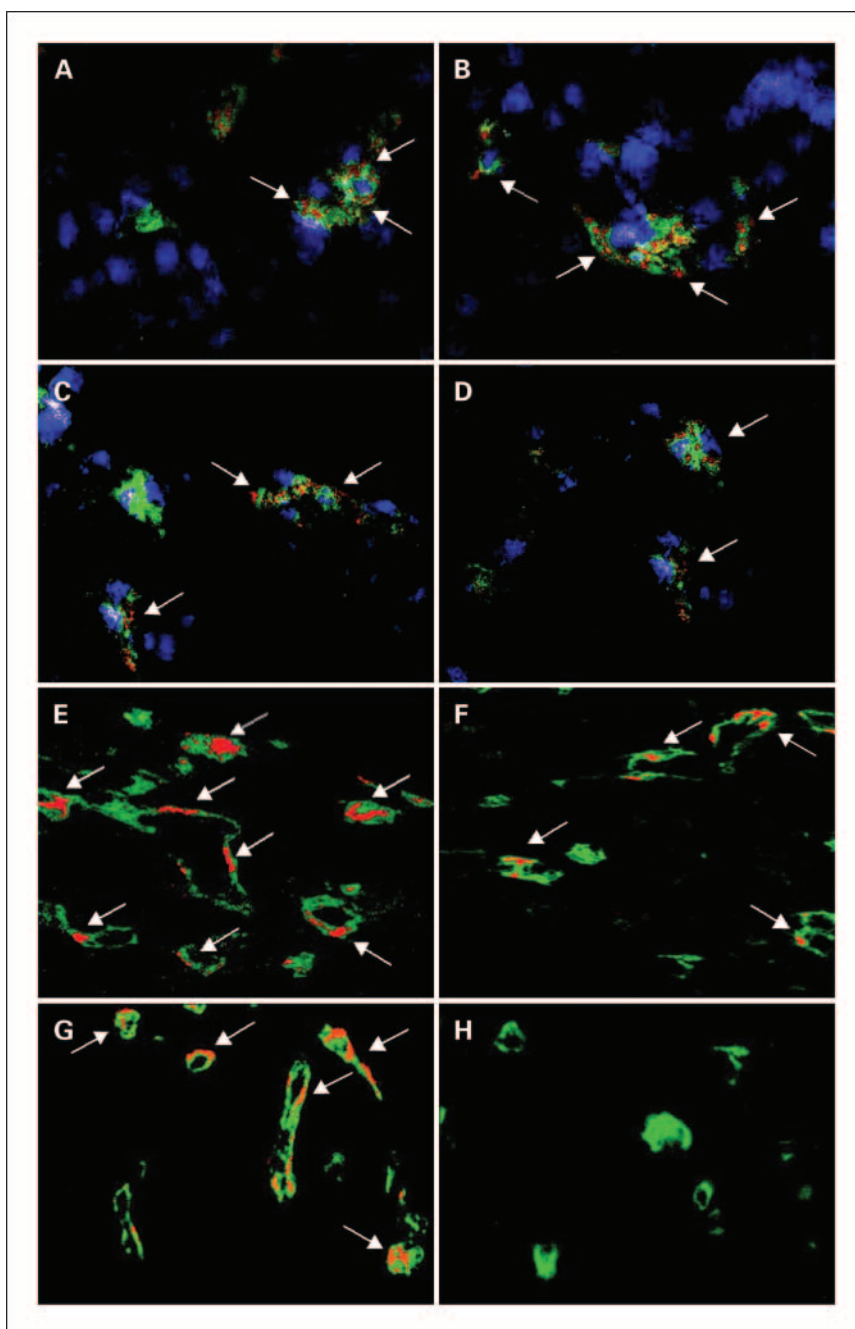


Fig. 6. Confocal microscopy analysis of CD133 and endothelial cell markers on bone marrow endothelial cells and neovessels. Some FVIII-RA⁺ (A), VEGFR-2⁺ (B), VE-cadherin⁺ (C), and Tie2/Tek⁺ (D) clustered MMECs (green stained) coexpress CD133 (red stained; arrows). CD133⁺ HSPCs are shown in the neovessel wall together with FVIII-RA⁺ (E), VEGFR-2⁺ (F), and VE-cadherin⁺ (G) MMECs (arrows). H, MGUS patient with no CD133 expression inside the FVIII-RA⁺ vessels.

Emerging evidence indicates that bone marrow-derived circulating EPCs can contribute to tumor angiogenesis and growth of certain tumors (3). A higher number of EPCs have been shown in the bone marrow of patients with active MM than in those with treated MM or with MGUS, or of healthy subjects (24), which reflects the increased angiogenic potential in MM. Zhang et al. (8) have assessed the view that EPC level and function are correlated with MM activity. Data from 31 patients showed that levels of circulating EPCs were higher in patients than in healthy subjects and that the cell VEGFR-2 expression was also higher in MM, suggesting that this molecule plays a critical role in the emergence and mobilization of EPCs in this tumor.

Here, we isolated CD133⁺CD34⁺ HSPCs from peripheral blood of MM patients before conditioning to transplant. These cells displayed very low VEGFR-2 expression unlike the circulating EPCs isolated by Zhang et al. (8) and the circulating endothelial cells isolated by Rigolin et al. (25). Compared with the latter, these HSPCs lacked the VE-cadherin expression. When exposed to VEGF, FGF-2, and IGF, these HSPCs differentiated into mature endothelial cells because they progressively lost CD133 and acquired the expression of FVIII-RA, VEGFR-2, and VE-cadherin, which are typical markers of mature endothelial cells (19, 26, 27). VEGFR-1 was acquired too and probably mediates HSPC recruitment and migration (28). On day 21 of culture, the expression of these endothelial

cell markers overlapped in differentiated HSPCs and resting paired bone marrow MMECs. Furthermore, HSPCs generated a capillary-like network similarly to MMECs. Overall, these data show for the first time that, in MM patients, circulating HSPCs are driven by VEGF, FGF-2, and IGF to become mature endothelial cells through a vasculogenic pathway, much in the same way as normal bone marrow and circulating and cord blood HSPCs (26, 27).

In bone marrow biopsies of patients with MM, but not MGUS, we observed some CD133⁺ HSPCs in the constitution of the vessel wall of newly forming blood vessels together with FVIII-RA⁺, VEGFR-2⁺, and VE-cadherin⁺ MMECs. We hypothesize that in MM patients plasma cells and inflammatory cells secrete high levels of VEGF, FGF-2, and IGF, which recruit bone marrow and circulating HSPCs into the tumor microenvironment bed (7), where they differentiate into MMECs and participate in the formation of the neovessel wall. On the contrary, because in MGUS these cytokines are secreted at very low levels (29), HSPCs remain in a resting state; hence, they cannot participate in the neovessel formation. Grunewald et al. (30) have shown that adult vasculogenesis may be induced by the recruitment of bone marrow-derived circulating cells by secretion of VEGF from the microenvironment. Moreover, these VEGF-recruited cells are predominantly hematopoietic in nature and home to the tumor perivascular sites.

About 10% of bone marrow MMECs express CD133 (10) that can derive from HSPCs engaged in the endothelial differentiation via vasculogenesis. These differentiating HSPCs interact with classic MMECs involved in neovessel wall building via angiogenesis. Thus, like in solid tumors (31–33), neovessels may be formed in MM through angiogenesis combined with vasculogenesis.

After purification with the anti-CD133 antibody, HSPCs from the bone marrow of MM patients express very low VEGFR-2 levels, confirming observations in murine models (34), and do not express VE-cadherin and FVIII-RA. HSPC expression of VEGFR-2 and VE-cadherin is crucial for their endothelial cell differentiation. In fact, VEGFR-2-deficient mice lack vessel

formation (13, 15–17, 19). VE-cadherin expression, an inevitable step in HSPC differentiation along the endothelial cell lineage (20), is needed for interendothelial cell contacts and consequently for neovascular assembly (35). Loss of CD133 expression as VEGFR-2 expression increases implies HSPC differentiation into endothelial cells (5, 22), as shown here in MM patients. Our observations, together with evidence from other sources that HSPCs differentiate into endothelial cells and other mesodermal cell types (5, 22, 23, 36), lead us to speculate that in HSPC cultures a multipotent cell population expressing low VEGFR-2 levels corresponds to the EPC precursor described by Zhang et al. (8) and, in turn, it seems to be the MMEC precursor.

Grant et al. (37) observed that transplanting single HSPCs into mice that were subsequently subjected to retinal injury, some neovessels after injury were derived from donor HSPCs. Moreover, Cogle et al. (38), using the same retinal model, showed that human donor HSPCs contributed to the murine host vasculature.

In conclusion, in MM patients, we show for the first time that HSPC-derived endothelial cells have phenotypic and functional characteristics of the mature endothelium. In the MM bone marrow microenvironment, we postulate plasma cells and inflammatory cells to provide angiogenic stimuli that recruit HSPCs into the tumor bed and induce their differentiation into endothelial cells and participate directly in the formation of the vessel wall, thus contributing to tumor vasculature. Our data confirm that HSPCs in bone marrow and peripheral blood can contribute to the new blood vessel formation in a process similar to vasculogenesis (5, 22, 23) and suggest that VEGF and FGF-2 inhibitors may be useful in the antivascular management of MM.

Acknowledgments

We thank Milena Rizzi for her technical assistance, Prof. Nazareno Capitanio for critical revision of the manuscript, and Dr. Geraldine A. Boyd for editing the English version of this manuscript.

References

- Risau W, Flamme I. Vasculogenesis. *Annu Rev Cell Dev Biol* 1995;11:73–91.
- Risau W. Mechanisms of angiogenesis. *Nature* 1997;386:671–4.
- Ribatti D. The discovery of endothelial progenitor cells. An historical review. *Leuk Res* 2007;31:439–44.
- Yin AH, Miraglia S, Zanjani ED, et al. AC133, a novel marker for human hematopoietic stem and progenitor cells. *Blood* 1997;95:5002–12.
- Peichev M, Naiyer AJ, Pereira D, et al. Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. *Blood* 2000;95:952–8.
- Vacca A, Ribatti D. Bone marrow angiogenesis in multiple myeloma. *Leukemia* 2006;20:193–9.
- Ribatti D, Nico B, Vacca A. Importance of the bone marrow microenvironment in inducing the angiogenic response in multiple myeloma. *Oncogene* 2006;25:4257–66.
- Zhang H, Vakili V, Braunstein M, et al. Circulating endothelial progenitor cells in multiple myeloma: implications and significance. *Blood* 2005;105:3286–94.
- Ria R, Falzetti F, Ballanti S, et al. Melphalan alone ver-
- us melphalan plus busulphan in conditioning to autologous stem cell transplantation for low-risk multiple myeloma. *Haematol J* 2004;5:118–22.
- Vacca A, Ria R, Semeraro F, et al. Endothelial cells in the bone marrow of patients with multiple myeloma. *Blood* 2003;102:3340–8.
- Vacca A, Scavelli C, Serini G, et al. Loss of inhibitory semaphorin 3A (SEMA3A) autocrine loops in bone marrow endothelial cells of patients with multiple myeloma. *Blood* 2006;108:1661–7.
- Medvinsky A, Dzierzak E. Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell* 1996;86:897–911.
- Peault B. Hematopoietic stem cell emergence in embryonic life: developmental hematology revisited. *J Hematother* 1996;5:369–78.
- Fong GH, Zhang L, Bryce DM, Peng J. Increased hemangioblast commitment, not vascular disorganization, is the primary defect in flt-1 knock-out mice. *Development* 1999;126:3015–25.
- Choi K, Kennedy M, Kazarov A, Papadimitriou JC, Keller G. A common precursor for hematopoietic and endothelial cells. *Development* 1998;125:725–32.
- Choi K. Hemangioblast development and regulation. *Biochem Cell Biol* 1998;76:947–56.
- Shalaby F, Rossant J, Yamaguchi TP, et al. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* 1995;376:62–6.
- Gehling UM, Ergun S, Schumacher U, et al. *In vitro* differentiation of endothelial cells from AC133-positive progenitor cells. *Blood* 2000;95:3106–12.
- Ribatti D, Vacca A, Nico B, Ria R, Dammacco F. Cross-talk between hematopoiesis and angiogenesis signaling pathways. *Curr Mol Med* 2002;2:537–47.
- Nishikawa SI, Nishikawa S, Kawamoto H, et al. *In vitro* generation of lymphohematopoietic cells from endothelial cells purified from murine embryos. *Immunology* 1998;8:761–9.
- Yamashita J, Itoh H, Hirashima M, et al. Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. *Nature* 2000;408:92–6.
- Rafii S, Shapiro F, Rimarachin J, et al. Isolation and characterization of human bone marrow microvascular endothelial cells: hematopoietic progenitor cell adhesion. *Blood* 1994;84:10–9.
- Lin Y, Weisdorf DJ, Solovey A, Hebbel RP. Origins of circulating endothelial cells and endothelial outgrowth from blood. *J Clin Invest* 2000;105:71–7.

24. Dominici M, Campioni D, Lanza F, et al. Angiogenesis in multiple myeloma: correlation between *in vitro* endothelial colonies growth (CFU-En) and clinical-biological features. *Leukemia* 2001;15:171–6.
25. Rigolin GM, Fraulini C, Ciccone M, et al. Neoplastic circulating endothelial cells in multiple myeloma with 13q14 deletion. *Blood* 2006;107:2531–5.
26. Melero-Martin JM, Khan ZA, Picard A, Wu X, Paruchuri S, Bischoff J. *In vivo* vasculogenic potential of human blood-derived endothelial progenitor cells. *Blood* 2007;109:4761–8.
27. Metharom P, Caplice NM. Vascular disease: a new progenitor biology. *Curr Vasc Pharmacol* 2007;5:61–8.
28. Chen Y, Amende I, Hampton TG, et al. Vascular endothelial growth factor promotes cardiomyocyte differentiation of embryonic stem cells. *Am J Physiol Heart Circ Physiol* 2006;291:1653–8.
29. Di Raimondo F, Azzaro MP, Palumbo G, et al. Angiogenic factors in multiple myeloma: higher levels in bone marrow than in peripheral blood. *Haematologica* 2000;85:800–5.
30. Grunewald M, Avraham I, Dor Y, et al. VEGF-induced adult neovascularization: recruitment, retention, and role of accessory cells. *Cell* 2006;124:175–89.
31. Xiong-Zhi W, Dan C, Guang-Ru X. Bone marrow-derived cells: roles in solid tumor. *Neoplasia* 2007;54:1–6.
32. Bruno S, Bussolati B, Grange C, et al. CD133⁺ renal progenitor cells contribute to tumor angiogenesis. *Am J Pathol* 2006;169:2223–5.
33. Li B, Sharpe EE, Maupin AB, et al. VEGF and PlGF promote adult vasculogenesis by enhancing EPC recruitment and vessel formation at the site of tumor neovascularization. *FASEB J* 2006;20:1495–7.
34. Ziegler BL, Valtieri M, Porada GA, et al. KDR receptor: a key marker defining hematopoietic stem cells. *Science* 1999;285:1553–8.
35. Menon C, Gharthey A, Canter R, Feldman M, Fraker DL. Tumor necrosis factor- α damages tumor blood vessel integrity by targeting VE-cadherin. *Ann Surg* 2006;244:781–91.
36. Reyes M, Lund T, Lenvik T, Aguiar D, Koodie L, Verfaillie CM. Purification and *ex vivo* expansion of post-natal human marrow mesodermal progenitor cells. *Blood* 2001;98:2615–25.
37. Grant MB, May WS, Caballero S, et al. Adult hematopoietic stem cells provide functional hemangioblast activity during retinal neovascularization. *Nat Med* 2002;8:607–12.
38. Cogle CR, Wainman DA, Jorgensen ML, Guthrie SM, Mames RN, Scott EW. Adult human hematopoietic cells provide functional hemangioblast activity. *Blood* 2004;103:133–5.