Vascular Biology, Atherosclerosis and Endothelium Biology

Circulating Monocytes Expressing CD31

Implications for Acute and Chronic Angiogenesis

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To identify the roles of various circulating cells (eg, endothelial and/or stem and progenitor cells) in angiogenesis, we parabiosed a wild-type syngeneic mouse with a transgenic syngeneic green fluorescent protein mouse. Following the establishment of a common circulation between these parabionts, we investigated acute (7 to 10 days), subacute (2 to 3 weeks), and chronic (4 to 6 weeks) phases of angiogenesis in wild-type mice using wound healing, implanted gel foam fragments, and subcutaneous tumor assays, respectively. We found that under *in vitro* conditions, circulating murine monocytes expressed F4/80, CD31, and vascular endothelial growth factor receptor 2, but neither CD133 nor von Willebrand factor, whereas murine endothelial cells expressed CD31, vascular endothelial growth factor receptor 2, and von Willebrand factor, but neither CD133 nor F4/80. Immunofluorescence analysis revealed that green fluorescent protein-positive cells in the walls of new vessels in wounds, gel foam blocks, and tumors expressed both F4/80 and CD31, that is, macrophages. Pericytes, cells that express both CD31 and desmin, were found both in the walls of tumor-associated vessels and within tumors. Collectively, these data demonstrate that monocytes (ie, cells that express both CD31 and F4/80) may be recruited to the site of tissue injury and directly contribute to angiogenesis, reaffirming the close relationships between various cell types within the reticuloendothelial system and suggesting possible targets for anticancer treatments. (*Am J Pathol* 2009, 174:1972–1980; DOI: 10.2353/ajpath.2009.080819)

The progressive growth of neoplasms and establishment of cancer metastasis depend on the development of adequate vasculature, ie, angiogenesis.^{1–4} The identification of critical factors that contribute to angiogenesis is a major goal of antivascular therapy.^{5,6} Whether postnatal neovascularization results from the proliferation and migration of endothelial cells of pre-existing blood vessels^{7–9} or from circulating stem and progenitor cells that are mobilized from the bone marrow and differentiate into mature endothelial cells^{10–15} has been controversial. Circulating endothelial cells (CD31+) have been reported to participate in blood vessel formation occurring during physiological and pathological processes, such as inflammation, wound healing, cardiovascular diseases, and cancer,^{9–18} and these circulating cells have been targets of cancer therapy.^{19–22} While several investigators have concluded that tumor-associated blood vessels consist of 50% bone marrow-derived endothelial cells,^{23–25} others have reported that the contribution of circulating bone marrow-derived endothelial cells was either low or undetectable.^{26,27} One possibility to account for these differences could be the mice under study and their general state of health. In several studies reporting the participation of circulating CD31+ cells in angiogenesis, mice were given lethal x-irradiation or a high dose of chemotherapy leading to myeloablation and then reconstituted with green fluorescence protein (GFP)-labeled bone marrow cells. Whether the same pattern of angiogenesis occurs in physiological conditions is unclear.

To directly investigate the contribution of circulating endothelial cells to the establishment of neovasculature in normal mice, we used the same technique that ruled out the assumption that ovulated oocytes in adult mice are derived from circulating germ cells,²⁸ ie, we examined the formation of blood vessels in wounds, implanted

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gel-foam sponges, and subcutaneous tumors in mice surgically joined by parabiosis.^{29,30} Specifically, we joined a genetically marked GFP mouse with a normal mouse. The parabiosed mice developed a common circulation,^{28–31} which allowed us to track genetically marked cells passing from one parabiont to the other and to determine whether these circulating cells contributed to the establishment of blood vessels in healing wounds, gel foam sponges, and subcutaneous tumors.

Materials and Methods

Animals

Female transgenic green fluorescent mice (C57BL/6-Tg [ACTB-EGFP]10sb/J) were purchased from the Jackson Laboratory (Bar Harbor, ME). The mice were housed and maintained under specific-pathogen-free conditions. Age-matched female C57BL/6 mice were purchased from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research Facility (Frederick, MD). Our animal facilities were approved by the American Association for Accreditation of Laboratory Animal Care and met all current regulations and standards of the United States Department of Agriculture, United States Department of Health and Human Services, and the NIH. The mice were used in accordance with institutional guidelines when they were 8 to 10 weeks old.

Parabiosis

A transgenic female green fluorescent mouse and a female C57BL/6 (wild-type) mouse were anesthetized with pentobarbital (0.5 mg/g body weight) (Abbott Laboratories, Chicago, IL). Opposing sides of the bodies were shaved. The skin was cleaned with iodine and alcohol and then was widely excised from the shoulder (humerus) joint to the hip (femur) joint. The muscle layer was separated by meticulous dissection. The two mice were then anastomosed as follows: the muscle layer of each mouse was approximated by interrupted absorbable sutures (4-0 vicryl; Ethicon, Inc., Somerville, NJ) and then was sutured skin to skin by continuous non-absorbable suture (4-0 prolene; Ethicon, Inc.). Additional continuous anchoring suture to secure the anastomosis was placed over the anastomosis site. The parabionts were wrapped with elastic band to relieve wound tension.

Confirmation of Common Circulation

Establishment of common circulation was confirmed by injecting Evans blue dye³¹ into one mouse, followed by direct imaging of fluorescence under the fluorescent microscope or the peripheral blood smear of the wild-type mouse. Two weeks after parabiosis, pairs of parabionts were randomly selected. Evans blue was injected into the tail vein of one of the parabionts. Staining of the partner mouse with blue color proved the establishment of a common circulation. We also observed the parabiosed mice under a fluorescent microscope. The presence of

fluorescent signals in the wild-type mice was documented and compared with that in transgenic green fluorescent mice. Peripheral blood smears were prepared from the wild-type mice by tail bleeding. The presence of fluorescent cells confirmed the establishment of a common circulation. Wound assay, gel foam assay, and tumor assays were then performed.

Mouse Endothelial Cells, Circulating Monocytes, and Macrophages

We determined the expression of CD31, F4/80, vascular endothelial growth factor receptor (VEGFR2), CD133, and von Willebrand factor on mouse endothelial cells, macrophages, and circulating monocytes. For the collection of mouse macrophages, 2 ml of thioglycolate (Sigma, St. Louis, MO) was injected intraperitoneally, and macrophages were collected 72 hours later by peritoneal lavage with Hanks' balanced salt solution.³² Mouse lung, brain, and prostate endothelial cells were established from a *H-2Kb-tsA58* mouse.³³

First, we performed Western blot to confirm the specificity of antibodies as well as determine the expression of markers on mouse endothelial cells, macrophages, and circulating monocytes. Briefly, whole-cell lysates of mouse brain endothelial cells, lung endothelial cells, prostate endothelial cells, and macrophages were prepared using 1 ml of lysis buffer (10 mmol/L Tris [pH 8.0], 1 mmol/L EDTA, 0.1% SDS, 1% deoxycholate, 1% NP40, 0.14 M/L NaCl, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin, and 1 μ g/ml pepstatin) containing a protease inhibitor mixture (Roche, Indianapolis, IN). Samples containing equal amounts of protein (30 μ g) were separated by electrophoresis on 4 to 12% Nu-PAGE gels (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes. After blocking with TTBS (TBS + 0.1% Tween 20) containing 5% nonfat milk, the membranes were incubated at 4°C overnight with mouse monoclonal antibody against CD31 (PECAM-1, 1:1000, Pharmingen, San Diego, CA), mouse monoclonal antibody against F4/80 (1:1000, Serotec, Raleigh, NC), mouse monoclonal antibody against CD133 (1:1000, Abcam, Cambridge, MA), rabbit polyclonal antibody against von Willebrand factor (1:500, Abcam), rabbit monoclonal antibody against VEGFR2 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), and mouse monoclonal antibody against β -actin (Sigma, St. Louis, MO). Blots were then exposed to horseradish peroxidase-conjugated secondary antibodies (1:3000) and visualized by the enhanced chemiluminescence system from Amersham (Piscataway, NJ). Equal protein loading was confirmed by stripping the blots and reprobing them with an anti- β -actin antibody. Next, mouse lung endothelial cells and mouse macrophages were plated on chamber slides and stained for CD31³⁴ and F4/80³⁵ as described below.

In the last set of experiments, the phenotype of circulating monocytes was studied using immunofluorescent staining and confocal microscopy. Whole blood was collected from mice and the red blood cell fraction was lysed with a solution consisting of 0.15 M/L NH₄Cl, 1.0 mmol/L KHCO₃, and 0.1 mmol/L EDTA in water (pH 7.3).

Cells were centrifuged at 200 rpm, washed once in PBS, and then fixed in 2% paraformaldehyde for 20 minutes. The cell number was adjusted to 1.0×10^6 cells/ml, and 200 μ l of the suspension was spun onto slides using a Shandon Cytospin 3 (Shandon, Inc., Pittsburgh, PA). The cells were spun onto the slides over a period of 5 minutes (at 800 rpm), after which the slides were stored in acetone at 4°C. The following primary antibodies were used for staining: rat anti-CD31 (Pharmingen), rabbit anti-CD133 (Abcam), rabbit anti-VEGFR2 (Santa Cruz Biotechnology), and rabbit anti-von Willebrand factor (Abcam). Before labeling the cells, low-affinity IgG receptors were blocked for 20 minutes with a CD16/CD32 receptor antibody (Pharmingen). Samples were incubated with either primary antibody (1:100) or isotype-matched control antibody for 1 hour at ambient temperature. After this time period, the samples were washed twice, then labeled with the appropriate Cy3 secondary antibody (Molecular Probes, Carlsbad, CA) for 45 minutes. Samples were then rinsed, blocked, and incubated with a 1:100 dilution of F4/80-Alexa647 conjugated antibody (Serotec) in blocking solution consisting of 5% horse serum and 1% goat serum in PBS. Samples were viewed using a Zeiss LSM 510 laser scanning microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with an argon laser (458/477/488/514 nm, 30 mW), HeNe laser (413 nm, 1 mW, and 633 nm, 5 mW), LSM 510 control and image acquisition software, and appropriate filters (Chroma Technology Corp., Brattleboro, VT). Three days later, the skin and wound area were harvested and frozen in OCT (Sakura Fineter, Torrance, CA). Sections were analyzed by immunofluorescence for expression of CD31, F4/80, CD133, and von Willebrand factor.

Gel Foam Angiogenesis Assay

The development and optimization of the gel foam assay were described in detail previously.³⁶ Briefly, gel foam sponges (Pharmacia & Upjohn, Peapack, NJ) were cut into $0.5 \times 0.5 \times 0.5$ cm cubes and soaked overnight at 4°C in PBS. The saturated sponges were then soaked in a 1:1 mixture of 1% ultrapure agarose (Invitrogen Corp., Carlsbad, CA) and PBS or PBS containing basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and interleukin-8 (IL-8). The final working concentration of cytokines was 2 μ g/ml. After hardening at room temperature for 1 hour, the sponges were implanted subcutaneously into wild-type mice or transgenic green fluorescent mice as described previously. After 2 to 3 weeks, the gel foam sponges were harvested and frozen in OCT. Frozen sections were analyzed by immunohistochemistry for expression of CD31, F4/80, CD133, and von Willebrand factor.

Tumor Vasculature

Mouse Lewis lung carcinoma (3LL) cells (1×10^5 in 200 μ l of Hanks' balanced salt solution) were injected subcutaneously into the dorsum of wild-type mice. When the tumor reached 10 mm in diameter (4 to 6 weeks), tumors

were removed and frozen in OCT. Sections were analyzed by immunofluorescence for expression of CD31, F4/80, CD133, and von Willebrand factor.

Immunofluorescence Double Staining for CD31, F4/80, CD133, von Willebrand Factor, and Desmin

Frozen tissues in OCT were sectioned (6 to 8 μ m), mounted on positively charged slides, and air-dried for 30 minutes. The sections were fixed in cold acetone for 10 minutes and then washed three times with PBS for 3 minutes each. Double staining for CD31 and F4/80 required a protein block with 5% normal horse serum and 1% normal goat serum for 5 minutes. Rat anti-mouse CD31 monoclonal antibody (PECAM-1, Pharmingen, San Diego, CA) was applied at a 1:400 dilution in blocking solution. After overnight incubation at 4°C, the slides were washed with PBS 3 times for 3 minutes. Sections were blocked for 5 minutes at room temperature, and goat anti-rat Cy5 antibody (GxRt-Cy5) (Jackson ImmunoResearch, West Grove, PA) was added at a 1:600 dilution and incubated in blocking solution for 1 hour at room temperature. The slides were then washed three times for 3 minutes with PBS. To prevent cross-reaction, sections were incubated with rat IgG isotype (1:100 dilution) in blocking solution (H&L, Jackson ImmunoResearch) for 1.5 hours at room temperature and then washed three times for 3 minutes with PBS. Fragment block was then performed with Fab Block (GxRt) IgG (1:50 dilution; Jackson ImmunoResearch) in blocking solution for 1.5 hours at room temperature, and sections were then washed with PBS three times for 3 minutes. Sections were blocked for 5 minutes and incubated with rat anti-mouse F4/80 monoclonal antibody (1:100 dilution; Serotec, Raleigh, NC) in blocking solution overnight at 4°C. Tissue sections were then washed three times with PBS for 3 minutes. Protein block was performed for 5 minutes, and goat anti-rat Cy-3 antibody (GxRt-Cy3) (Jackson ImmunoResearch) was added at a 1:1000 dilution and incubated in blocking solution for 1 hour at room temperature. Double immunofluorescence staining of CD31 or F4/80 with VEGFR2 (1:200), CD133 (1:200), or von Willebrand factor (1:500) was performed without fragment block procedures by Fab Block. Goat anti-rat Cy5 antibody (1:600, Jackson ImmunoResearch) for labeling CD31 and F4/80, and goat anti-rabbit Cy3 antibody (1:600, Jackson ImmunoResearch) for VEGFR2, CD133, and von Willebrand factor were used as the secondary antibodies. The slides were washed three times for 3 minutes with PBS, and nuclei were stained at room temperature for 10 minutes with Hoechst 33342 (Molecular Probe, Eugene, OR). The slides were then washed three times with PBS for 3 minutes each. Coverslips were placed on top of the slides using glycerol in PBS with fluorescent mounting medium (propyl gallate, Acros Organics, Morris Plains, NJ).

To identify pericytes, double staining for CD31 and desmin was performed.²⁷ Slides were incubated with goat anti-mouse IgG, Fab fragment (Jackson ImmunoResearch) overnight at 4°C to block endogenous immuno-

globulin, followed by incubation with protein blocking solution for 5 minutes. The slides were then incubated with mouse anti-desmin antibody (1:200 dilution, Molecular Probe) in protein blocking solution for 8 hours at 4°C. The slides were washed three times for 3 minutes each with PBS

and then treated with protein blocking solution for 5 minutes. Slides were incubated with Cy3-conjugated secondary antibody (Jackson ImmunoResearch) and then stained for CD31 as described above. Images were captured with an Olympus microscope (BX-51) with an attached DP71 digital camera and processed with DP Controller and DP-Manager software (Olympus, Center Valley, PA).

Results

Establishment of Common Circulation

Two weeks after parabiosis, a green fluorescent signal could be directly visualized in the ear and foot pad of a wild-type mouse where vessels are merged (Figure 1A). Injection of Evans Blue dye stained the injected mouse and the parabiont partner. Injected Evans Blue was excreted in the urine of the injected mouse and the parabiont partner (Figure 1B). Green fluorescent cells were detected in the peripheral blood smear collected from the wild-type mouse (Figure 1C).

In Vitro Expression of CD31, F4/80, CD133, VEGFR2, and von Willebrand Factor on Mouse Endothelial Cells, Mouse Macrophages, and Mouse Circulating Monocytes

Using Western blot analysis, we detected expression of CD31 and VEGFR2 in mouse endothelial cells (lung, brain, prostate) and macrophages. Expression of F4/80 was detected only in mouse macrophages and expression of von Willebrand factor was detected only in mouse endothelial cells. CD133 was not expressed in mouse endothelial cells or mouse macrophages (Figure 2A). For double immunofluorescence staining, we plated mouse lung endothelial cells and mouse macrophages in chamber slides and stained them with rat anti-mouse CD31 or rat anti-mouse F4/80 antibody. Images were captured for CD31 and/or F4/80. Nuclei were stained blue. Peritoneal macrophages expressed F4/80 and CD31. Lung endothelial cells expressed only CD31 (Figure 2B). Confocal analysis of double immunofluorescence staining revealed that, in agreement with previous reports,^{37,38} circulating monocytes expressed F4/80, CD31, and VEGFR2, but not CD133 or von Willebrand factor. Color codes are red for F4/80, green for nucleus, and blue for CD31, VEGFR2, CD133, and von Willebrand factor (Figure 2C).

In Vivo Wound Assay

The data in Figure 3A show that vessel walls in the healing wounds consisted of red-staining cells (CD31-

Figure 1. Establishment of common circulation between wild-type and green fluorescent mouse. Two weeks after parabiosis, mice were observed under the fluorescent microscope. **A:** Green fluorescent signals were detected on the various parts of the wild-type mouse. **B:** Two weeks after parabiosis, Evans Blue dye was injected into one mouse, and the counterpart mouse was also stained blue. Injected Evans Blue dye was excreted in the urine of both mice. **C:** In peripheral blood smear of the wild-type mouse, circulating green fluorescent cells were observed.

Figure 2. Western blot analysis for expression of CD31, F4/80, CD133, VEGFR2, and von Willebrand factor in mouse endothelial cells and mouse macrophages. **A:** von Willebrand factor was expressed in mouse endothelial cells. F4/80 was expressed in mouse macrophages. Expression of CD133 was not detected in either mouse endothelial cells or mouse macrophages. **B:** Immunohistochemical analysis of mouse macrophages and mouse lung endothelial cells for expression of CD31 and F4/80. Mouse macrophages and lung endothelial cells were plated on the chamber slides and stained for CD31 and F4/80. Mouse macrophages expressed CD31 and F4/80 but mouse lung endothelial cells expressed only CD31. **C:** Double Immunofluorescence staining of F4/80 and CD31, VEGFR2, CD133, or von Willebrand factor. Circulating mouse monocytes express F4/80, CD31, and VEGFR2 on their surface but not CD133 or von Willebrand factor. Color codes are red for F4/80, green for nucleus, and blue for CD31, VEGFR2, CD133, or von Willebrand factor.

positive cells of the wild-type mouse), pink-staining cells (CD31- and F4/80-positive cells from the wild-type mouse), and white-staining cells (CD31- and F4/80-positive cells from the transgenic mouse). Colocalization immunofluorescence analysis identified the cells infiltrating into the healing to be macrophages of the wild-type mouse (blue) and macrophages of the transgenic mouse (sky blue).

Gel Foam Assay

Although gel foam fragments enriched with bFGF, VEGF, or IL-8 contained more blood vessels than control gel foam fragments, we did not find any differences in the composition of cells within the fragments. Colocalization immunofluorescence analysis revealed that the majority of the cells in the wall of new vessels within the gel foam stained pink or white, ie, they were macrophages from the wild-type or transgenic mouse. Red-

staining cells (CD31-positive cells from wild-type mouse) were found only in vessels in the periphery of the gel foam (data not shown). Vessels in the center of the gel foam contained pink-staining cells (CD31- and F4/80-positive cells from the wild-type mouse) and white-staining cells (CD31- and F4/80-positive cells from the transgenic mouse) (Figure 3B). Endothelial cells of transgenic mice (green stain) expressing CD31 (red) should stain yellow. We did not find any yellow-staining cells in any of the vessels. Blue-staining cells (F4/80-positive from the wild-type mouse) and green-staining cells (negative for CD31 or F4/80 from the transgenic mouse) were found in the gel foam. We did not identify any red cells (CD31+).

Tumor Assay

Colocalization immunofluorescence analysis revealed that the walls of large vessels were composed of cells

A Wound

B Gel foam

C Tumor

GFP (+)	● (cells from GFP mouse)
CD31 (+)	● (CD31+ cells from wild type mouse)
F4/80 (+)	● (F4/80+ cells from wild type mouse)
CD31(+)/F4/80(+)	● (CD31+ and F4/80+ cells from wild type mouse)
GFP(+)/CD31(+)	● (CD31+ cells from GFP mouse)
GFP(+)/F4/80(+)	● (F4/80+ cells from GFP mouse)
GFP(+)/CD31(+)/F4/80(+)	○ (CD31+ and F4/80+ cells from GFP mouse)

Figure 3. Immunofluorescence analysis of circulating cells participating in angiogenesis. **A:** Wound images of green (GFP+ cells), red (CD31), and blue (F4/80) were merged. In the wound angiogenesis assay, vessel walls were composed of cells that stained red (CD31-positive cells from wild-type mouse), pink (CD31- and F4/80-positive cells from wild-type mouse), and white (CD31- and F4/80-positive cells from green fluorescent mouse). Infiltration by macrophages of wild-type mouse (blue) and green fluorescent mouse (sky blue) were observed. **B:** In the periphery of gel foam, CD31-positive (red) and CD31- and F4/80-positive cells of wild-type mouse (pink) and green fluorescent mouse (white) were found in the vessel walls. In the middle of gel foam, where only newly formed vessels were present, only CD31- and F4/80-positive cells of wild-type mouse (pink) or green fluorescent mouse (white) were found. **C:** In tumor lesions, vessels were composed of CD31-positive cells of wild-type mouse (red), CD31- and F4/80-positive cells of wild-type mouse (pink), and green fluorescent mouse (white).

that stained red (CD31-positive cells from the wild-type mouse), pink (CD31- and F4/80-positive cells from the wild-type mouse), and white (CD31- and F4/80-positive cells from the transgenic mouse) (Figure 3C). In small blood vessels, we found pink-staining (F4/80 and CD31) and white-staining (GFP, F4/80, and CD31) cells. Yellow cells (CD31-positive cells of the transgenic mouse) were extremely rare. Cells that stained blue (F4/80 cells from the wild-type mouse), green (CD31- and F4/80- negative cells from the transgenic mouse), and sky blue (F4/80-positive cells from the transgenic mouse) were identified in the non-vascular portions of the subcutaneous tumor.

Expression of CD31 and Desmin

Wound Assay

Desmin was labeled blue. Cells infiltrating the healing wound or cells in walls of blood vessels did not express desmin (Figure 4A). Wall of vessels consisted of red cells (CD31-positive cells from wild-type mouse) or yellow cells (CD31-positive cells from green fluorescent mouse). We did not find desmin-positive cells in the 7-day-old vessels.

Gel Foam Assay

Desmin expression was not found in cells participating in angiogenesis in the gel foams (Figure 4B). CD31-positive cells from the wild-type mouse (red) or CD31-

positive cells from the green fluorescent mouse (yellow) composed the vascular walls.

Tumor Assay

Desmin-positive cells were located on the outer layer of vessel walls in the 4- to 6-week-old tumor vasculature, and desmin-positive cells from the wild-type mouse (blue) or transgenic mouse (sky blue) were also found in the tumor tissue. The lumen of tumor-associated blood vessel walls consisted of CD31-positive cells from the wild-type mouse (red). The outer layer of vessels had few yellow-staining cells (CD31+ and GFP+) and desmin-positive cells from the wild-type mouse (blue) or transgenic mouse (sky blue). CD31- and desmin-positive cells from the wild-type mouse (pink) or transgenic mouse (white) were found on the outer layer of some vessels (Figure 4C).

Expression of CD31, F4/80, CD133, and von Willebrand Factor

F4/80 and CD31 were labeled red, CD133 and von Willebrand factor were labeled green, and nuclei were stained blue. In wounds or gel foam, we did not find cells expressing CD133 or von Willebrand factor (Figures 5, A and B). Tumor cells and cells in the wall of large vessel in tumors stained positive for von Willebrand factor. Cells in the wall of newly developed small vessels did not express von

A Wound

B Gel foam

C Tumor

- GFP(+)
- CD31(+)
- Desmin(+)
- CD31(+)/Desmin(+)
- GFP(+)/CD31(+)
- GFP(+)/Desmin(+)
- GFP(+)/CD31(+)/Desmin(+)
- (cells from GFP mouse)
- (CD31+ cells from wild type mouse)
- (Desmin+ cells from wild type mouse)
- (CD31+ and Desmin+ cells from wild type mouse)
- (CD31+ cells from GFP mouse)
- (Desmin+ cells from GFP mouse)
- (CD31+ and Desmin+ cells from GFP mouse)

Figure 4. Immunofluorescence analysis for expression of CD31 and desmin. Tissues were stained for CD31 and desmin. Desmin positive cells were not found in wound (A) or gel foam (B) tissues. C: CD31- and desmin-positive cells of wild-type mouse (pink) and green fluorescent mouse (white) were found in vessels of tumor tissues. Infiltration of desmin-positive cells of wild-type mouse (blue) and green fluorescent mouse (sky blue) were also found in tumors.

Willebrand factor (Figure 5C), implicating that those vessels could include recruited monocytes. CD133 was expressed on cells in wounds as well as on cells surrounding hair follicles. CD31-positive cells did not express CD133 (Figure 5D).

Discussion

The pre-existing vascular network cannot provide adequate oxygen and nutrients to inflammatory lesions, healing wounds, ischemia from damage to vessels, and neo-

A

Wound

F4/80/von Willebrand factor

B

Gel foam

F4/80/CD133

C

Tumor

CD31/von Willebrand factor

D

Wound

CD31/CD133

Figure 5. Double immunofluorescence staining for F4/80 or CD31 with CD133 or von Willebrand factor. F4/80-positive cells (red) did not express von Willebrand factor (green), or CD133 in wound (A) or gel foam (B), respectively. CD31-positive cells (red) in the wall of large tumor vessels did not express von Willebrand factor (C). Mouse Lewis lung carcinoma cells express von Willebrand factor (C). Cells in the wound lesion and cells surrounding hair follicles were positive for CD133 (green). CD31-positive cells (red) were not co-localized with CD133 (green) (D).

plasmas;³⁹ therefore, the maintenance of cell viability requires the formation of new vasculature, ie, angiogenesis.³² Endothelial cells in new vessels have been assumed to originate from dividing differentiated endothelial cells of the local vasculature,^{1,3,4} mononuclear cells,⁴⁰ and bone marrow-derived circulating endothelial cells.⁴¹ However, whether circulating cells can give rise to the luminal endothelium layer or the damaged intima⁴² has been controversial.^{37,38,43–45} As mentioned previously, many of the experiments demonstrating that circulating CD31+ endothelial cells can give rise to new vessels used transplantation of bone marrow cells to animals receiving massive doses of x-irradiation or chemotherapy. Whether these animals can be used as a model for nonmyeloablative settings is unclear.

To determine whether circulating CD31+ cells can contribute to the development of new blood vessels, we used the parabiosis model, which recently ruled out the assumption that ovulated oocytes in adult mice are derived from circulating germ cells.²⁸ The common circulation established between the wild-type C57BL/6 mouse and the transgenic GFP C57BL/6 mouse provided us with a system with physiological cellular hemo-dynamics²⁹ to study acute (wound assay of 7 to 10 days), subacute (gel foam implant assay of 14 to 21 days), and chronic (subcutaneous tumor of 4 to 6 weeks) phases of angiogenesis. Detailed immunohistochemical analysis allowed us to determine whether circulating cells that express GFP (transgenic mouse origin) contributed to the formation of blood vessels in the wild-type mouse.

Colocalization immunofluorescence analysis clearly showed that circulating blood macrophages (F4/80+) also express CD31. In contrast, vascular endothelial cells expressed only CD31 (Figure 2). CD31+ cells (stained red) originating from transgenic mice (green color) should stain yellow (green plus red). We did not detect any yellow staining cells in the lumen of vessels of wounds, gel foam plugs, or tumors (Figure 3). The luminal cells stained red, indicating that they were CD31+ endothelial cells from the wild-type mouse. The gel foam implants contained numerous blood vessels that developed after implantation of the fragments. All of the vessels found within the gel foam implants consisted of cells expressing CD31 (red) and CD31 plus F4/80 of the wild-type mouse (pink = CD31+/F4/80+ from wild-type mouse) and CD31+/F4/80+ cells from the transgenic mouse (white) (Figure 3B).

The presence of macrophages in damaged endothelium and the migration of macrophages through walls of vessels have been known for decades.⁴⁶ Thus, our finding that macrophages localized in the vessel wall as well as in the tumor stroma is not surprising. Circulating monocytes are most likely to be first recruited to sites of tissue damage and developing vessels that originate from proliferating endothelial cells of pre-existing vessels. Whether macrophages are essential to initiate angiogenesis should therefore be investigated. Mature blood vessels are recognized by the presence of pericytes (desmin-positive) that originate from circulating cells,⁴⁷ and our data show that GFP-positive, CD31-positive, and desmin-positive cells (white stain) can

indeed be found in the outer wall of vessels of subcutaneous tumors.

In conclusion, the clear demonstration that monocytes that express F4/80 also express CD31 suggests that evidence relying on identification of circulating endothelial cells merely on the expression of CD31 should be re-examined. Our data clearly confirm the close relationship between reticular cells and endothelial cells.⁴⁸

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