Circulating Vascular Progenitor Cells Do Not Contribute to Compensatory Lung Growth

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Abstract—The biological principles that underlie the induction and process of alveolization in the lung as well as the maintenance of the complex lung tissue structure are one of the major obstacles in pulmonary medicine today. Bone marrow–derived cells have been shown to participate in angiogenesis, vascular repair, and remodeling of various organs. We addressed this phenomenon in the lung vasculature of mice in a model of regenerative lung growth. C57BL/6 mice were transplanted with bone marrow from one of three different reporter gene transgenic strains. flk-1/lacZ mice, tie-2/lacZ transgenic mice (both exhibiting endothelial cell–specific reporter gene expression), and ubiquitously enhanced green fluorescent protein (eGFP)-expressing mice served as marrow donors. After hematopoietic recovery, compensatory lung growth was induced by unilateral pneumonectomy and led to complete restoration of initial lung volume and surface area. The lungs were consecutively investigated for bone marrow–derived vascular cells by lacZ staining and immunohistochemistry for phenotype identification of vascular cells. lacZ- or eGFP-expressing bone marrow–derived endothelial cells could not be found in microvascular regions of alveolar septa. Single eGFP-positive endothelial cells were detected in pulmonary arteries at very low frequencies, whereas no eGFP-positive vascular smooth muscle cells were observed. In conclusion, we demonstrate in a model of lung growth and alveolization in adult mice the absence of significant bone marrow–derived progenitor cell contribution to the concomitant vascular growth and remodeling processes. (Circ Res. 2003;93:372-379.)

Key Words: stem cells ▪ plasticity ▪ vascular endothelium ▪ vascular smooth muscle ▪ alveolization

Many recent reports present evidence for unexpected plasticity of bone marrow–derived stem or progenitor cells.1–11 The transplantation of whole bone marrow or enriched hematopoietic or mesenchymal stem cells was shown to lead to the integration of progeny into blood vessels and to their differentiation into endothelial cells and perivascular cells in different organs under physiological or pathological conditions.3,12–14 Bone marrow–derived endothelial cells were found to contribute to vascular growth and repair in adult mammals, a phenomenon that was termed postnatal vasculogenesis.12,15 Significant integration of bone marrow–derived vascular cells was shown in animal models of cardiac infarction16 and stroke.17,18 Furthermore, endothelial cells from circulating progenitors have been shown to significantly contribute to tumor angiogenesis.19 The derivation of endothelial cells and hematopoietic cells from one proximal stem cell, the so-called hemangioblast, has been observed in embryonic tissues20 and recently at sites of vascular growth in adult animals.21 The integration of bone marrow–derived vascular cells into growing or aging blood vessels is not only of biological interest per se but would give rise to new therapeutic concepts of vascular diseases.

Loss of functional lung tissue attributable to a variety of lung diseases is already one of the main causes of mortality in industrial societies.22,23 Chronic obstructive pulmonary disease and emphysema are now the twelfth leading cause of disability and will become the third leading cause of death in industrial countries in 2025. It is therefore of utmost importance to gain better insight into the mechanisms and regulation of maintenance, repair, and regeneration of the pulmonary gas exchange surface. The mechanisms that control the onset, rate, and cessation of alveolus formation as well as the confounding epithelial and pulmonary vascular growth and differentiation are minimally understood.24 Pulmonary angiogenesis and endothelial survival are critical for alveolus formation and homeostasis.25,26 Compensatory lung growth is an ideal model to investigate processes and programs of alveolization in adult mammals.27–31 The contribution of bone marrow–derived cells to growth, repair, and maintenance of the lung vascular system is unknown. In this study, we specifically addressed this question for the first time by using animal models of bone marrow transplantation with marrow derived from one of three different transgenic donors that
express reporter genes under endothelial cell–specific or ubiquitously active promoters to investigate whether pulmonary endothelial and perivascular cells derive from circulating progenitors. C57BL/6 wild-type mice were transplanted with bone marrow of mice that express the lacZ reporter gene under the endothelial cell–specific flk-1 promoter (vascular endothelial growth factor receptor 2) or tie-2 (angiopeptin receptor) promoters. Bone marrow from ubiquitous enhanced green fluorescent protein (eGFP)-expressing mice was used as a control to identify all bone marrow–derived cells after bone marrow transplantation into wild-type mice. The transplanted cells and their progeny could later be detected in the lung tissue because of their reporter gene expression. In wild-type mice, which were bone marrow–transplanted with the endothelial cell–specific lacZ reporter gene–expressing cells, only bone marrow–derived differentiated endothelial cells will express lacZ, whereas transplantation of the eGFP-transgenic marrow allows for the detection of progeny by GFP expression in all tissues independently of the cellular differentiation.

Materials and Methods

Bone Marrow Transplantation and Transgenic Mice

C57BL/6J or C57BL/6 wild-type mice 12 to 16 weeks of age were lethally irradiated with 11.0 Gy, and 2 to 5 × 10^6 transgenic bone marrow cells were transplanted. Bone marrow was harvested by flushing tibiae and femurs of 8- to 12-week old mice with RPMI 1640 containing 1% FCS, 100 U/mL penicillin, and 1000 U/mL streptomycin. The first group of transgenic donor mice was C57BL/6-TgN(ActbEGFP)1Osb (Jackson Laboratory, Bar Harbor, Maine). The third group of transgenic donor mice was tie-2/lacZ mice derived from circulatory endothelial and perivascular cells derived from embryogenesis and adulthood. Each group was bone marrow–transplanted with the endothelial cell–specific lacZ reporter gene–expressing cells, only bone marrow–derived differentiated endothelial cells will express lacZ, whereas transplantation of the eGFP-transgenic marrow allows for the detection of progeny by GFP expression in all tissues independently of the cellular differentiation.

Animal Surgery

Mice underwent left-sided pneumonectomy 6 to 8 weeks after BMT. The mice were anesthetized with an intraperitoneal injection of 60 mg/kg ketamine and 2 mg/kg xylazine, orally intubated, and mechanically ventilated with a mouse ventilator (Hugo Sachs Elektronik, March-Hugstetten). The left lung was carefully lifted through an incision in the 6th intercostal space, tied at the hilus, and resected. The animals recovered in a warmed cage with chow and water provided ad libitum. The animal handling and study protocol conformed with the guidelines for animal experiments of the University of Giessen and were approved by the local authorities for animal ethics and animal experiments.

Histological Analysis of Mice Transplanted With eGFP Transgenic Marrow

The animals were euthanized with halothane. The lung was inflated with a pressure of 10 cm H_2O and perfusion fixed with 1% buffered paraformaldehyde (Sigma-Aldrich) with a pressure of 25 cm H_2O. The lungs were postfixed overnight in 1% PFA at 4°C, dehydrated over a graded series of alcohol, and paraffin embedded. Sections of 4 to 10 μm were cut on a microtome (Leica). Antigen retrieval was performed by incubation with trypsin solution (Digest All 2, Zymed) for 10 minutes at 37°C. Antibody staining was performed following standard procedures. All incubations and washes were done with PBS + 2.5% calf serum plus 0.1% Triton X-100. Unspecific binding sites were blocked over 30 minutes with PBS containing 2.5% calf serum and 10% goat serum (Sigma-Aldrich). Incubation times for primary and secondary antibodies were 60 and 30 minutes, respectively. The following were used as primary antibodies: CD3 (clone MEC13.3; dilution 1:100), CD34 (clone MEC14.7; 1:100; both Pharmingen), CD45 (1:50; Cymbus Biotechnologies), α-smooth muscle actin (1:400) and vimentin (1:200; both Sigma-Aldrich), von Willebrand factor (1:400; Dako), anti-β-galactosidase (1:1000; Cortex), and anti-GFP (1:400; Abcam, Cambridge, UK). Tissue staining with the biotinylated griffonia simplificolia isoelectin B4 (5 μg/mL; Sigma-Aldrich) was performed according to Hellstrom et al. Anti–flk-1 antibody was provided by Dr S. Nishikawa (Department of Microbiology, Kyoto Prefectural University of Medicine, Kyoto, Japan). As secondary antibodies, goat anti–rat-Cy3, streptavidin-Cy3 (1:1000), goat anti–rabbit-FITC (1:100; all Pharmingen) or goat anti–rat-alexa488, and goat anti–rabbit-alexa555 (1:2000; Molecular Probes) were used. The sections were examined with a Leica TCS confocal microscope (Leica) using the 488-nm line of the Argon laser. Fluorescent signals from eGFP/FITC and Cy3 were viewed simultaneously in separate detector channels. True color overlays of single and serial sections were generated with the Leica confocal software.

Histological Analysis of Mice Transplanted With lacZ Transgenic Marrow

OCT compound was instilled intratracheally, and the lungs were embedded in OCT and shock frozen. Cryosections were obtained on a cryostat (Leica), air dried, and fixed with 2% PFA for 20 minutes at room temperature. X-gal staining was done as described previously. Each staining was controlled by parallel staining of a lung section from a wild-type mouse (negative control) and the flk-1/lacZ donor mouse (positive control). The slides were viewed with a Zeiss Axioscope microscope (Zeiss).

Whole-Mount lacZ Staining

Perfusion-fixed lungs were placed in X-gal staining solution at 30°C overnight. Afterward, the lungs were washed three times in PBS, dehydrated over a graded series of ethanol, and cleared in a 1:2 solution of benzyl alcohol and benzyl benzoate (Sigma) to transparency for macroscopic assessment under a stereomicroscope (Zeiss Stemi SV11).

Determination of Lung Volume

Lungs were instillation-fixed with 4% paraformaldehyde plus 0.1% glutaraldehyde in PBS for 1 hour with a pressure of 20-cm water column. Lung volume was measured by fluid displacement according to the method of Scherle.

Results

Compensatory Lung Growth

We partially characterized compensatory lung growth in C57BL/6 mice by quantification of lung volume and alveolar surface area after left-sided pneumonectomy. Volumes of right lungs 21 days after pneumonectomy were measured and compared with the total volumes of left and right lungs of nonoperated control mice. Lung volumes were normalized to individual body mass (mass-specific volume). Mass-specific volumes of right lungs 21 days after pneumonectomy (30.6±0.9 cm³/g) did not significantly differ from mass-specific volumes of controls (29.7±0.9 cm³/g, Figure 1). Furthermore, mass-specific alveolar surface of both groups did not differ as well, which proves substantial alveolization...

Transplantation With flk-1/H11001/lacZ or tie-2/lacZ Transgenic Marrow and Consecutive Compensatory Lung Growth

To investigate bone marrow–derived endothelial cells in pulmonary vessels after regenerative lung growth, wild-type mice were transplanted with bone marrow of either flk-1/H11001/lacZ or tie-2/lacZ transgenic mice, which express the lacZ reporter gene specifically in endothelial cells (Figures 2b and 2d through 2f). Endothelial expression of β-gal in flk-1/H11001/lacZ mice as well as flk-1 and tie-2 in adult postpneumonectomy lungs was shown by specific antibody staining (Figures 2a through 2d). After the reconstitution of the bone marrow, the mice were unilaterally pneumonectomized to evoke rapid growth of lung tissue and pulmonary vessels. Four weeks after the pneumonectomy, when compensatory lung growth was completed, histological analysis of the lungs failed to reveal integrated, lacZ-positive bone marrow–derived endothelial cells in the lungs of all animals of either the flk-1/H11001/lacZ or the tie-2/lacZ transplanted group (Figures 2g and 2h). At least 10 50-μm thick sections of each lung were examined without counterstain to ensure even the detection of single, weakly lacZ-positive cells (pictures not shown). lacZ expression of integrated endothelial cells could neither be found in alveolar septa nor in larger vessels. Whole-mount lacZ staining of the lungs of two flk-1/H11001/lacZ and two tie-2/lacZ bone marrow–transplanted wild-type mice 21 days after pneumonectomy, performed to view the whole lungs for the identification of regional clusters of bone marrow–derived endothelial cells, confirmed the absence of lacZ-positive cells (Figure 3).

To control successful bone marrow engraftment, polymerase chain reaction (PCR) for β-galactosidase was performed on the bone marrows of the transplanted animals and was positive in all cases (Figures 4a and 4b). Furthermore, lacZ staining of bone marrow showed lacZ-positive cells, demonstrating that flk-1–driven and tie-2–driven reporter gene expression was detectable in transplanted animals (Figures 4c and 4d).
Transplantation With eGFP Transgenic Marrow Followed by Consecutive Compensatory Lung Growth

To additionally substantiate the lacZ-transgenic marrow experiments, we used eGFP-transgenic marrow transplantation that allowed for the identification of all progeny irrespective of the cellular differentiation. The success of the transplantation with eGFP transgenic bone marrow was assessed by flow cytometric analysis for eGFP fluorescence of the mononuclear cells from peripheral blood. Six weeks after transplantation, >80% of peripheral blood of the transplanted animal was GFP-positive (Figures 5a through 5d), a number that increased to >90% after 6 months. Flow cytometric analysis for differentiation markers of lymphocytes, monocytes, and granulocytes revealed a normal hematopoietic system at 6 weeks. Single GFP-positive cardiomyocytes were detected at time of tissue sampling, possibly representing engraftment of bone marrow–derived cells in this tissue (Figures 5e and 5f). Paraffin sections of 4-μm thickness were immunostained for endothelial antigens and examined for colocalization of GFP-positive bone marrow–derived cells and the endothelial markers by use of conventional fluorescence microscopy. Because of the utmost proximity of endothelial cells and intravascular leukocytes in alveolar septa, a definitive distinction of both cell types in these regions without accepting many false-positive events was in our hands not possible with this standard technique (Figure 6a). To gain maximal resolution, we investigated 10-μm sections with a confocal laser scan microscope. For each antigen investigated, at least three sections of each lung were stained. Histological analysis of the eGFP-transplanted mice revealed abundant bone marrow–derived cells in alveolar regions as well as in peribronchial and perivascular regions. The shapes of the bone marrow–derived cells in alveolar regions varied from small rounded morphology to large, delicately elongated cells with thin protrusions, which stretched sometimes over more than one alveolar septum. Most of the alveolar macrophages were GFP-positive at this time point (10 to 12 weeks after bone marrow transplantation). Endothelial cell immunostaining with anti-CD31, anti-CD34, anti–von Willebrand factor, and GSI-B4 lectin revealed absence of colocalization of these markers and GFP-fluorescence in alveolar regions (Figures 6b through 6f).

Rarely, single bone marrow–derived cells with either morphologically or immunologically determined endothelial phenotype were found to be integrated in the intimal layer of pulmonary arteries (≈1 cell in every 10th section, Figures 7a through 7d). Virtually all GFP-positive cells in alveolar, peribronchial, and perivascular regions stained positive for the pan-leukocyte marker CD45. Even elongated cells in the alveolar septa, which could have been judged by morphology to be endothelial or epithelial cells, all expressed CD45 and were consequently considered leukocytes, most probably resembling dendritic cells (Figures 7e and 7f). Furthermore, groups of bone marrow–derived leukocytes were detected peribronchially at branching points of the airways, the location of the bronchus-associated lymphatic tissue. Staining for α-smooth muscle actin and vimentin, performed to detect putative differentiation of bone marrow–derived cells into smooth muscle cells or fibroblasts, revealed complete absence of GFP-positive smooth muscle or fibroblastic cells in all slides investigated (pictures not shown).
Discussion

We investigated the contribution of bone marrow–derived precursor cells to pulmonary vascular growth and maintenance in adult mice. To induce substantial lung growth in adult animals, we applied a model of compensatory lung growth that follows left-sided pneumonectomy and leads to total restoration of lung volume, alveolar number, alveolar surface, and cellular number in small rodents.\(^2\)\(^7\),\(^2\)\(^8\) Compensatory lung growth in adult mice restored initial lung volume (Figure 1) and gas exchange area over 21 days and therefore represents an excellent model for the investigation of alveolization and pulmonary vascular growth. In this study, adult wild-type C57BL/6 mice underwent bone marrow transplantation with transgenic marrows containing endothelial cell–specific\(^3\)\(^2\),\(^3\)\(^3\) (flk-1\(^{+/H11001}\)/lacZ or tie-2/lacZ) or ubiquitous reporter gene expression (cac/eGFP).\(^3\)\(^4\) After hematopoietic recovery, the left lung was resected. The right lungs were removed 3 weeks later and examined for bone marrow–derived vascular cells. In mice transplanted with flk-1\(^{+/H11001}\)/lacZ or tie-2/lacZ transgenic bone marrow, lacZ expression was completely absent in large vessels as well as in pulmonary capillaries of the alveolar septa after pneumonectomy, which suggests that bone marrow–derived endothelial cells do not participate in compensatory lung growth. We can exclude false-negative results attributable to unsuccessful bone marrow transplantation, because PCR for the lacZ transgene was strongly positive in bone marrow samples of all transplanted animals at the time of tissue sampling (10 to 12 weeks after transplantation), indicating that a significant amount of donor cells engrafted. Furthermore, cells staining positive for lacZ were detected in bone marrow of the transplanted animals, which was not the case in controls, and served as an internal positive controls of bone marrow cell engraftment into structural organ tissues. Confocal laser scan microscopy: panel e is a reconstruction of 20 sections through a 7-\(\mu\)m slice; panel f is a single section. Scale bars = 25 \(\mu\)m.
growth or regeneration of the lung. The positive findings of other groups, which investigated vessel growth under pathological conditions in heart, liver, and brain, do not necessarily imply the same biologic phenomenon to be a cause of lung regeneration as well. Many of these investigations applied bone marrow or stem cell injections as a therapy for experimental tissue damage. Our experiments were not designed as a way of applying stem cells therapeutically at the time of an organ damage like ischemia or inflammation but rather to investigate the proportion of bone marrow contribution to a process of regulated regenerative growth without interfering with endogenous circulating stem cell concentrations. Recent studies suggested differences in stem cell recruitment attributable to different organ lesions.

Based on these findings it is still possible that in varying models of lung disease, bone marrow–derived cells may play a significant role.

In comparable models, bone marrow–derived cells were investigated in organ maintenance over 12 months and after a bleomycin-induced lung lesion. Both groups reported significant integration of progeny and differentiation into pulmonary epithelial type 2 or type 1 cells, respectively. The comparably high bone marrow–derived epithelial cell type 2 number in the lung was discussed to be attributable to the high pulmonary irradiation sensitivity that could in return provoke substantial epithelial regeneration. Additionally, the continuous growth of mice during their lifetime could contribute to the ongoing generation of lung tissue. Endothelial cells, on the other hand, are very sensitive to irradiation, and

Figure 6. Phenotypic characterization of GFP-expressing bone marrow–derived cells in lungs of mice by staining for endothelial cell markers. Conventional fluorescence microscopy for coexpression of GFP (green fluorescence) and endothelial markers (red) derived colocalization of both colors for many cells in alveolar septa as shown for CD34 (a, 4-μm paraffin section). These colocalization signals could not be confirmed using confocal laser scan microscopy for the readout of CD34 (b), CD31 (c and d), von Willebrand factor (e), and GSI-B4 lectin expression (f). All eGFP-expressing cells in alveolar septa were intravascular or perivascular cells that did not express endothelial markers. d and f, Reconstructions of 20 confocal sections through 7 μm to show more clearly the polymorphic shape or intravascular localization of these cells, respectively. Scale bar=25 μm.

Figure 7. Bone marrow–derived endothelial cells in pulmonary vessels after compensatory lung growth represent a very rare event. Rarely, bone marrow–derived endothelial cells (green fluorescence) were detected in the intimal layer of pulmonary arteries. Confocal microscopy images of a GFP-expressing cell stained for CD31 is shown in panel a (arrow). A rather small cell expressing GFP and binding GSI-B4 lectin is shown in panel b. Cells of endothelial cell appearance based on morphology [elongated cell and nuclear shape, direct contact to lamina elastica interna [arrowheads]] are shown in sections stained for α-smooth muscle actin (c) and lycopersicon esculentum lectin (d). Phenotypic characterization for the pan-leukocyte antigen CD45 (e and f) showed that virtually all bone marrow–derived cells in the lung represented hematopoietic cells despite their sometimes very intriguing cellular shapes that might have suggested structural parenchymal cell types. c, e, and f, Reconstructions of 20 confocal sections through 7-μm tissue depth. Scale bar=25 μm.
endothelial damage contributes to pneumonia following irradiation of the lung.\textsuperscript{41,42} We therefore assumed that endothelial repair after irradiation as well as the vascular growth concomitant to the compensatory lung growth would be a stimulus for the integration of endothelial precursor cells. The fact that we could not show this phenomenon in our experiments may be attributable to the comparably shorter time course of our experiments, where the tissues were investigated 10 to 12 weeks after bone marrow transplantation. However, the repair and regenerative growth of the lungs are completed by then, and it is unlikely that a substantial contribution of donor cells to the endothelial or perivascular cell pool will be found later on. Recent investigations by Wagers et al.\textsuperscript{43} questioned earlier results of abundant stem cell contribution to the lung tissue maintenance and the integration of bone marrow–derived pulmonary epithelium. Nevertheless, it may be of great but yet unknown importance which kinds of stem cells are used, if they went through purification or cell culture steps, or if they are of hematopoietic or mesenchymal phenotype.

In conclusion, we investigated the contribution of bone marrow–derived cells to pulmonary angiogenesis and vascular remodeling by applying a model of lung growth and alveologization in adult mice. Using three different transgenic mice strains as bone marrow donors, we provide evidence for the failure of bone marrow–derived vascular precursor cells to significantly contribute to the generation of endothelial cells, pericytes, vascular smooth muscle cells, and fibroblasts in postsenonecotomy lung growth in the adult mouse, which implies that the proliferative capacity of endogenous cell compartments of the lung would be sufficient for this process.

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