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Circ Res. 2003;93:372-379; originally published online July 24, 2003; doi: 10.1161/01.RES.0000087643.60150.C2

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Circulating Vascular Progenitor Cells Do Not Contribute to Compensatory Lung Growth

Robert Voswinckel, Tibor Ziegelhoeffer, Matthias Heil, Sawa Kostin, Georg Breier, Tanja Mehlung, Rainer Haberberger, Matthias Clauss, Andreas Gaumann, Wolfgang Schaper, Werner Seeger

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 tissues 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-132 (vascular endothelial growth factor receptor 2) or tie-233 (angiopoietin receptor) promoters. Bone marrow from ubiquitously enhanced green fluorescent protein (eGFP)-expressing mice34 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 wild-type mice 12 to 16 weeks of age were lethally irradiated with 11.0 Gy, and 2 to 5 × 10⁶ transgenic bone marrow cells were transplanted. Bone marrow was harvested by flushing tibias 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)1Osbg (Jackson Laboratory, Bar Harbor, Maine). These mice ubiquitously express eGFP, which leads to green fluorescence of all cells except erythrocytes and hair follicle cells.34 Success of bone marrow transplantation (BMT) was monitored by fluorescence of all cells except erythrocytes and hair follicle cells.34 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 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.53 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 simultaneously in separate detector channels. True color overlays of single and serial sections were generated with the Leica confocal software.

Whole-Mount lacZ Staining

Perfusion-fixed lungs were placed in X-gal staining solution at 30°C overnight.53 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.37

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/H11545/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 postpneumonecotomy lungs was shown by specific antibody staining (Figures 2a through 2d). After the reconstitution of the bone marrow, the mice were unilaterally pneumonecotomized to evoke rapid growth of lung tissue and pulmonary vessels. Four weeks after the pneumonecotomy, 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 pneumonecotomy, 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 GS1-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 were 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).

Figure 3. Absence of endothelial lacZ expression in lungs of mice after transplantation with flk-1lacZ or tie-2lacZ bone marrow and unilateral pneumonectomy. To detect focal areas of bone marrow–derived endothelial cell engraftment, lungs of mice transplanted with either flk-1lacZ or tie-2lacZ bone marrow were perfusion-fixed and whole mount–stained for lacZ expression and the tissue was cleared to translucence. A representative picture from an flk-1lacZ bone marrow–transplanted mouse is shown. Blue lacZ staining was absent in lung tissue viewed macroscopically and under a stereomicroscope as shown in panel a. The partially air-filled bronchial tree is visible through the cleared tissue (arrows). For comparison, whole-mount lacZ stained lung of an flk-1lacZ bone marrow donor mouse is shown in panel b, where high expression of lacZ (blue staining) can be found in bronchial epithelium (arrows) and lower levels of expression in endothelial cells delicately delineating the vessels (arrowheads). Scale bars=50 μm.

Figure 4. Positive PCR for genomic lacZ from bone marrow samples and lacZ expression in bone marrow cells of flk-1lacZ and tie-2lacZ transplanted mice prove successful for marrow engraftment. To assess the success of bone marrow engraftment, PCR for the genomic lacZ transgene was performed on bone marrow samples of wild-type mice transplanted either with flk-1lacZ knock-in bone marrow (a) or tie-2lacZ transgenic bone marrow (b) at the time of lung tissue sampling. lacZ PCR was strongly positive for all bone marrows, indicating substantial engraftment of transplanted cells. Positive (+) and negative (−) lacZ PCR controls are shown. As an additional internal control of bone marrow engraftment, some bone marrow cells stained positive for lacZ (arrows) in all transplanted mice. Representative sections of bone marrow from flk-1lacZ (c) and tie-2lacZ (d) transplanted mice are shown. Scale bar=100 μm.
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. Compen-

satory 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 alveol-

ization and pulmonary vascular growth. In this study, adult wild-type C57BL/6 mice underwent bone marrow transplan-

tation with transgenic marrows containing endothelial cell–specific (flk-1/H11001/lacZ or tie-2/lacZ) or ubiquitous reporter gene expression (cac/eGFP). 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 transplanta-

tion, 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 trans-

plantation), 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 tis-

sues. Confocal laser scan microscopy: panel e is a reconstruction of 20 sections through a 7-μm slice; panel f is a single section. Scale bars = 25 μm.

Figure 5. Positive controls for successful eGFP-transgenic bone marrow trans-

plantation. Representative flow cytometric data of peripheral blood cells derived from wild-type control mice (a) and wild-
type mice 6 weeks after bone marrow transplantation with eGFP transgenic marrow (b through d). Top, Dot plots of forward scatter (FSC) and green eGFP- fluorescence intensity (eGFP). Bottom, Respective histograms, where the trans-

planted mice present with at least 80% eGFP-positive cells in the circulation. Some eGFP-positive cardiomyocytes were detected in hearts of GFP bone marrow–transplanted mice after pneumono-

tomy (e and f). These may serve as internal positive controls of bone marrow cell engraftment into structural organ tis-

sues. Confocal laser scan microscopy: panel e is a reconstruction of 20 sections through a 7-μm slice; panel f is a single section. Scale bars = 25 μm.
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 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 pneumonitis after 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 of donor cells to the endothelial or perivascular compartments of the lung. 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 postpneumonectomy 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.

\section*{Acknowledgments}

This work was funded in part by the Max-Planck-Society. The authors thank Dr Urban Deutsch from the Max-Planck-Institute for Vascular Biology in Muenster for kindly providing the Tie-2/flacZ mice.

\section*{References}


