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Bone marrow production of lung cells: The impact of G-CSF, cardiotoxin, graded doses of irradiation, and subpopulation phenotype

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Abstract

Objective— Previous studies have demonstrated the production of various types of lung cells from marrow cells under diverse experimental conditions. Our aim was to identify some of the variables that influence conversion in the lung.

Methods— In separate experiments, mice received various doses of total-body irradiation followed by transplantation with whole bone marrow or various subpopulations of marrow cells (Lin^{-/+}, c-kit^{-/+}, Sca-1^{-/+}) from GFP⁺ (C57BL/6-TgN[ACTbEGFP]10sb) mice. Some were given intramuscular cardiotoxin and/or mobilized with granulocyte colony-stimulating factor (G-CSF).

Results— The production of pulmonary epithelial cells from engrafted bone marrow was established utilizing green fluorescent protein (GFP) antibody labeling to rule out autofluorescence and deconvolution microscopy to establish the colocalization of GFP and cytokeratin and the absence of CD45 in lung samples after transplantation. More donor-derived lung cells (GFP⁺/CD45⁻) were seen with increasing doses of radiation (5.43% of all lung cells, 1200 cGy). In the 900-cGy group, 61.43% of GFP⁺/CD45⁻ cells were also cytokeratin⁺. Mobilization further increased GFP⁺/CD45⁻ cells to 7.88% in radiation-injured mice. Up to 1.67% of lung cells were GFP⁺/CD45⁻ in radiation-injured mice transplanted with Lin⁻, c-kit⁺, or Sca-1⁺ marrow cells. Lin⁺, c-kit⁻, and Sca-1⁻ subpopulations did not significantly engraft the lung.

Conclusions— We have established that marrow cells are capable of producing pulmonary epithelial cells and identified radiation dose and G-CSF mobilization as variables influencing the production of lung cells from marrow cells. Furthermore, the putative lung cell-producing marrow cell has the phenotype of a hematopoietic stem cell.

Introduction

There is a growing body of evidence to suggest that adult hematopoietic marrow cells have much more versatile differentiation capabilities than once believed. Numerous studies have now demonstrated the ability of adult stem cells to differentiate into a variety of cells from nonhematopoietic organs, including the skin [1], muscle [2,3], bone [4], heart [5], brain [6], liver [7,8], and lung [9–13]. In murine models, structurally and functionally normal pulmonary epithelial cells, including bronchial epithelial cells [9] and type I (AE I) [10] and type II

pneumocytes (AE II) [11], have been shown to be derived from exogenous marrow cells. These findings are exciting and of particular importance as a “normal” phenotype may be achieved in certain human pulmonary diseases (i.e., cystic fibrosis) if a relatively small number of normally functioning cells were to replace defective cells.

Injury is thought to be among the factors that influence the homing of marrow cells to the target organ and production of cells of the target organ. Intratracheal administration of bleomycin to the lung induces pulmonary fibrosis in mice and has served as a valuable injury model. AE I are particularly sensitive to bleomycin and are the first alveolar cells to be injured, while AE II have a more variable sensitivity [14]. Kotton et al. [10] demonstrated the appearance of donor-derived cells in the lungs of mice as soon as one day after an infusion of *LacZ*-labeled plastic-adherent cultured bone marrow cells following intratracheal bleomycin injury. Donor cells had the morphologic appearance and molecular phenotype of AE I. Other investigators have used radiation as a mode of lung injury. Alveolar epithelium damaged by radiation is repopulated as a result of AE II proliferation and conversion into AE I. Theise et al. [11] infused whole bone marrow cells into mice exposed to 1200 cGy of total-body irradiation (TBI). Within three days, their lungs appeared hypocellular, with breakdown of capillaries within alveolar septa and extravasation of erythrocytes into alveolar spaces. This effect peaked at day 5, at which time functioning, donor-derived AE II could be detected. Based on these and other studies, it would appear that injury is an essential catalyst that enables the production of lung cells from marrow; however, the degree of organ injury, the mode of injury used, and characteristics of the donor marrow cells may be among the multitude of factors that govern the extent of tissue replacement and the phenotype of newly produced cells.

We evaluated the role of a variety of factors in the production of lung cells from murine marrow cells. TBI was used not only to produce chimeric mice with exogenous marrow cells, but also as a mode of injury to the lung. In the present study, we show that higher doses of radiation lead to more blood cell chimerism, injury, and lung cell production.

Recombinant granulocyte colony-stimulating factor (G-CSF) is widely used to induce mobilization of blood precursors in different clinical settings such as chemotherapy-induced myelosuppression and peripheral blood stem cell recollection for autologous and allogeneic bone marrow transplantation [15]. It is the major growth factor responsible for regulating granulopoiesis and promoting the survival, proliferation, functional activation, and maturation of cells of the neutrophil lineage [15]. G-CSF has also been shown to increase the expression and function of adhesive receptors on the surface of both hematopoietic progenitors and stromal cells, including vascular cell adhesion molecule (VCAM-1) [15], potentially influencing the homing of marrow cells to certain tissues. We demonstrate here that G-CSF augments the homing of exogenous hematopoietic marrow cells to the radiation-injured mouse lung and production of lung cells.

Cardiotoxin, a component of cobra venom, causes an intense inflammatory reaction, resulting in muscle necrosis soon after direct injection. Its cytotoxic effect is thought to be mediated by its ability to competitively bind ATP, thus blocking the enzymatic activities of Na^+/K^+ - ATPase [16]. Cardiotoxin is also believed to mediate inflammation by interacting with glycosaminoglycans (GAG), which are abundantly expressed in the tissues of the cardiovascular system [17]. GAGs function as co-receptors that facilitate the proper presentation of growth factors and chemokines necessary for cell migration, adhesion, and differentiation during wound healing and embryogenesis [17]. Cardiotoxin also directly injures the lungs as it has been shown to decrease the dynamic compliance of the lungs, induce hypoxemia by increasing the shunt fraction, and elevate pulmonary arterial pressures when injected into dogs [18]. It appears to induce these physiologic derangements by stimulating hemolysis and vascular congestion and increasing vascular permeability in the lungs by

disrupting endothelial surfaces [19]. Our data indicate that this form of lung injury in irradiated mice has a minimal but statistically significant effect on the production of lung cells.

Cells with stem cell activity comprise only a very small percentage of the total number of cells present in unpurified whole bone marrow. Those with certain phenotypic characteristics, including the presence of stem cell antigen 1 (Sca-1) [20,21] and c-kit [21] or the absence of any cell markers (Lin⁻) [20,21] that would define the cell as being differentiated, appear to be especially enriched with stem cell activity. We also demonstrate that hematopoietic marrow cells with these phenotypic characteristics preferentially reconstitute the bone marrow of radiation- and cardiotoxin-injured mice and produce lung cells.

Methods

Experimental animals

Six- to 8-week-old female C57BL/6 (H2Kb) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and were used as bone marrow transplant (BMT) recipients. Animals were certified to be pathogen free, housed in our animal facility, and given ad libitum access to food and acid water. GFP-transgenic breeding pairs C57BL/6-TgN (ACTbEGFP)10sb were also purchased from The Jackson Laboratory. Animals were bred in our animal facility by mating heterozygous GFP⁺ mice, separated by using a UV light source, to C57BL/6 mice to produce GFP⁺ transgenic animals. Our model is a β -actin promoter GFP trans-genic. Homozygote animals die early. Other transgenics can be bred as homozygotes and we are evaluating these.

Isolation and preparation of whole bone marrow

Six- to 8-week-old female GFP⁺ mice were used as donors. Mice were anesthetized with halothane and sacrificed by cervical dislocation. Whole bone marrow (WBM) was obtained by flushing the femurs, tibias, and pelvic bones with sterile 1 $\times$ phosphate-buffered solution (PBS). Cells were centrifuged at 1300 rpm for 10 minutes at 4°C, then resuspended in sterile 1 $\times$ PBS. Cells were counted after staining an aliquot with crystal violet.

Isolation and preparation of whole bone marrow for lineage depletion

WBM was isolated and prepared as described above. Cells were passed through a 25-gauge needle to make a single-cell suspension, then lineage depleted (Lin⁻) by adding the following antibodies: antiTer119, B220, Mac-1, GR-1, CD4, and CD8. After 15 minutes of incubation on ice, Dynabead M450 anti-rat IgG (Dyna; Lake Success, NY, USA) was added and lineage-positive (Lin⁺) cells were removed by a magnetic column and saved. Details of lineage depletion have been previously published [22].

Isolation and preparation of Sca-1⁺ and SCA-1⁻ stem cell populations

WBM was isolated and prepared as described above. Biotin anti-mouse LY-6AE (Sca-1) (PharMingen, San Diego, CA, USA) was added to a final concentration of 1 μ g/10⁶ cells, then incubated for 30 minutes on ice. Next, 1 $\times$ PBS/5% heat-inactivated fetal calf serum (HIFCS) was added; then the solution was centrifuged at 350g for 10 minutes. This step was repeated. Streptavidin-allophycocyanin (Molecular Probes, Eugene, OR, USA) was added to a final concentration of 1 μ g/10⁶ cells and incubated for 20 minutes. Next, 1 $\times$ PBS/5% HIFCS was added and then the solution was centrifuged at 350g for 10 minutes. The cells were resuspended in 1 mL 1 $\times$ PBS/5% HIFCS, then passed through a 40- μ m filter. Propidium iodide (0.05 mg/mL) was added (1:1000 dilution) and cells were then separated by the use of a modular flow cytometer (Moflo) (Cytomation, Fort Collins, CO, USA) into Sca-1⁺ and Sca-1⁻ populations.

Isolation and preparation of c-kit⁺ and c-kit⁻ stem cell populations

WBM was isolated as described above. Allophycocyanin-conjugated anti-c-kit (Pharmingen, San Diego, CA, USA) was added to a final concentration of 1 $\mu\text{g}/10^6$ cells, then incubated for 30 minutes. Cells were separated into c-kit⁺ and c-kit⁻ populations by using the same procedures as described above.

Experiment 1: Bone marrow transplantation after radiation injury

Mice were exposed to a cumulative dose of 500, 900, or 1200 cGy TBI (or no TBI). A photon-producing linear accelerator (Elekta, Norcross, Georgia, USA) was used as a source of radiation. Mice that were exposed to 500 cGy received the entire dose in one round, while those exposed to 900 or 1200 cGy received evenly divided doses twice, 3 hours apart. One hour after TBI, recipients received 5×10^6 WBM cells from female GFP⁺ donors by tail vein injection. Mice were sacrificed 1 month or 3 months after BMT for tissue analysis.

Experiment 2: Bone marrow transplantation and mobilization with G-CSF after radiation injury

Mice were exposed to a cumulative dose of 900 cGy TBI as described above. One hour after TBI, recipients received 5×10^6 WBM cells from female GFP⁺ donors by tail vein injection. Starting at three weeks after BMT, mice were given one, two, or three rounds of subcutaneous G-CSF, 250 $\mu\text{g}/\text{kg}$ (or no G-CSF). One round consisted of daily injections of G-CSF for 5 consecutive days, followed by 5 days without G-CSF. Mice were sacrificed 9 weeks after BMT for tissue analysis.

Experiment 3: Bone marrow transplantation after radiation and cardiotoxin injury

Mice were exposed to a cumulative dose of 900 cGy TBI as described above. One hour after TBI, recipients received 5×10^6 WBM cells from female GFP⁺ donors by tail vein injection. Seven days after BMT, 10 mM of cardiotoxin (*Naja mossambica mossambica*, Sigma, St. Louis, MO, USA) was injected into the right tibialis anterior muscles of some mice. Pilot studies using intravenous cardiotoxin showed some toxicity to the animals. Initial experiments using intramuscular cardiotoxin showed an increase in the number of marrow-derived pulmonary cells in our model. Mice were sacrificed 4 weeks after TBI for tissue analysis. Four groups were created for these experiments: Group 1: – TBI, – cardiotoxin; Group 2: – TBI, + cardiotoxin; Group 3: 900 cGy TBI, – cardiotoxin; Group 4: 900 cGy TBI, + cardiotoxin.

Data from lung samples in group 3 (900 cGy, – cardiotoxin) could not be collected due to fixation problems with the lung tissue. As a result, data collected from experiments #1 and #2 were used for comparison.

Two additional mice were exposed to a cumulative dose of 900 cGy TBI and received 5×10^6 WBM cells as described above. Four weeks later, 10 mM cardiotoxin was injected into the right tibialis anterior muscle of both mice. Starting 2 days prior to the cardiotoxin injection, two rounds of G-CSF (250 $\mu\text{g}/\text{kg}$) were given as described above. Mice were sacrificed 8 weeks after TBI for tissue analysis as described above.

Experiment 4: Bone marrow transplantation with whole bone marrow and stem cell subpopulations after radiation and cardiotoxin injury

Mice were exposed to a cumulative dose of 500 cGy TBI and received BMT one hour after TBI in the following fashion: Group 1: WBM, 3×10^6 cells; Group 2: Lin⁻, 1.4×10^6 cells; Group 3: Lin⁺, 1.4×10^6 cells; Group 4: c-kit⁻, 2.16×10^5 cells; Group 5: c-kit⁺, 2.16×10^5 cells; Group 6: Sca-1⁻, 8×10^5 cells; Group 7: Sca-1⁺, 8×10^5 cells.

Isolation of these cell types from whole bone marrow yielded different numbers of viable cells due to differences in isolation techniques; however, the same number of cells were infused into mice of each comparison group (Lin⁻ vs Lin⁺, Sca⁻ vs Sca⁺, c-kit⁻ vs c-kit⁺). Seven days after BMT, 10 mM cardiotoxin was injected into the right tibialis anterior muscle of all recipients as described above. Mice were sacrificed 4 weeks after TBI for tissue analysis (Table 1, summary of experimental protocols).

Determination of peripheral blood chimerism

Peripheral blood was taken from each mouse by tail vein bleeding 1 day prior to sacrifice. Fifty μ L of blood was incubated for 10 minutes with 1.3 mL of ice-cold erythrocyte lysing solution (150 mM NH₄Cl, 10 mM NaHCO₃, 1 mM EDTA, pH 7.4), and resuspended in 1 $\times$ PBS/1% paraformaldehyde (Sigma, St Louis, MO, USA). Chimerism was determined by fluorescence-activated cell sorter (FACS) analysis. Total peripheral blood chimerism was assessed by calculating the percent of GFP⁺ cells to the total number of GFP⁺ and GFP⁻ cells after subtracting the background.

Lung harvest and fixation

The pulmonary vasculature was flushed with 4% paraformaldehyde/1 $\times$ PBS and the lungs inflated with 1.25 mL of the same solution. Lungs were then cut into smaller pieces, placed in 10 mL of ice-cold PLP (balanced phosphate solution with 2% paraformaldehyde, sodium m-periodate, and L-lysine) for 2 hours, and transferred to a 7% sucrose solution where they remained at 4°C overnight. The following day, they were placed into ethanol solutions of increasing concentrations for 3 hours and mineral spirits for 90 minutes. The pieces were placed in 60°C molten paraffin for 2 hours, embedded in paraffin, and stored at 4°C.

Histochemical and immunofluorescence staining

All tissue analysis was done on 5- μ m lung sections. Sections were deparaffinized in xylene and ethanol and stored in 1 $\times$ PBS until the time of staining. Routine hematoxylin and eosin (H & E) staining was performed on two or more sections from each experimental animal to assess histological evidence of injury. Sections were labeled with biotinylated anti-mouse CD45 (PharMingen, San Diego, CA, USA) alone or in combination with an epithelial marker (mouse anti-cytokeratin AE1/AE3 monoclonal antibody, Chemicon, Temecula, CA, USA). Labeling with CD45 alone and co-labeling for CD45 and cytokeratin was performed as follows.

–CD45/cytokeratin. An antigen retrieval step was necessary for cytokeratin labeling and was performed by incubating samples with Proteinase K (5 μ g/mL) for 5 minutes at 37°C. The blocking steps included a peroxidase, 0.3% H₂O₂/0.3% horse serum, avidin/biotin (Avidin/Biotin Blocking Kit, Vector, Burlingame, CA, USA), and mouse on mouse blocks (M.O.M. Kit, Vector, Burlingame, CA, USA). Cytokeratin and CD45 antibodies were applied to each sample and incubated for 2 hours at room temperature. The CD45 signal was amplified by using the TSA Biotin System (PerkinElmer Life Sciences, Boston, MA, USA) per product protocol. Secondary antibodies, AMCA avidin for CD45 (Vector, Burlingame, CA, USA) and goat anti-mouse IgG rhodamine for cytokeratin (Chemicon, Temecula, CA, USA), were applied to each sample and incubated at room temperature for 30 minutes. Specific labeling with CD45 and cytokeratin antibodies was confirmed by direct comparison with samples labeled with isotype and secondary-only controls. –CD45 only. Blocking steps included peroxidase, avidin, and biotin blocks as described above. CD45 was applied to each sample and incubated at 37°C for 60 minutes. No amplification step was performed. The secondary antibody for CD45, Texas red avidin, was applied to each sample and incubated at room temperature for 60 minutes.

Evaluation for autofluorescence

PLP-fixed and paraffin-embedded lung samples had numerous bright green cells that were visualized in the FITC channel by fluorescence microscopy and presumed to be GFP⁺ cells. Samples from the 900-cGy TBI group (experiment #1) were deparaffinized as described above, then analyzed by deconvolution fluorescence microscopy (Zeiss Axioplan 2 microscope, Carl Zeiss, Oberkochen, Germany) at room temperature and photographed at 40× magnification using the AxioVision software package (Carl Zeiss, Oberkochen, Germany). The same samples were then permeabilized with 0.2% Triton-X in PBS and blocked with 20% horse serum. Anti-green fluorescent protein rabbit IgG, Alexa Fluor 488 conjugate (Molecular Probes, Eugene, OR, USA) was then applied to samples for 1 hour at room temperature. The same areas that were photographed were then rephotographed using the same exposure conditions and a direct comparison between pre- and post-antibody labeling photographs was made. To prove that these cells were truly GFP⁺ and not bright green due to autofluorescence, samples were labeled with anti-green fluorescent protein rabbit IgG, Alexa Fluor 594 conjugate (Molecular Probes, Eugene, OR, USA) as described above. This antibody is directly conjugated to a red fluorochrome so that true GFP⁺ cells would appear green in the FITC channel and red in the rhodamine channel of our microscope. Samples analyzed included some from the 900-cGy TBI group (experiment #1), non-irradiated C57BL/6 mice, and irradiated (900 cGy TBI) C57BL/6 mice transplanted with 5×10^6 WBM from C57BL/6 mice. Samples were photographed as described above. Ten random high-power fields (100×) from five samples in each group were analyzed. Three-dimensional images were created of sample cells from a 15-layer (0.5 μm/layer) z-stack in order to demonstrate colocalization of red and green signals from GFP⁺ cells.

Counting of donor cells in the lung and statistics

Expression of GFP and antigens stained by different fluorescent-conjugated antibodies were visualized by deconvolution fluorescence microscopy as described above. Eight to 10 random 100× high-power fields from at least two sections over 100 μm apart of each sample were counted. Slides labeled with CD45 only were mounted in the anti-fade media Vectashield (Vector, Burlingame, CA, USA) with 0.4 μmol DAPI (4,6-Diamidino-2-phenylindole, Sigma, St. Louis, MO, USA), which labels DNA. Nucleated (DA-PI⁺) cells, GFP⁺ cells, and CD45⁺ and CD45⁻ cells were all counted. We have defined all DAPI⁺ cells that are both GFP⁺ and CD45⁻ to be donor bone marrow-derived lung cells. GFP⁺/CD45⁻ cells were expressed as a percentage of DAPI⁺ cells. Hematopoietic cells are of different size and demonstrate different CD45 staining patterns, as noted in our samples. Isotype controls for CD45 (biotinylated rat IgG 2Bκ) and secondary-only controls (Texas red avidin) were performed and were negative. Slides labeled with CD45 and cytokeratin were mounted in the anti-fade media Vectashield without DAPI as CD45 cells were labeled with a secondary antibody detected in the microscope's DAPI channel. Donor-derived lung cells (GFP⁺ and CD45⁻, the cell adjacent to the large arrow in Fig. 2D) that also labeled for cytokeratin were identified. Cells that were GFP⁺, CD45⁻, and cytokeratin⁺ were defined as being donor-derived lung epithelial cells. Three-dimensional images were created of sample cells from a 15-layer (0.5 μm/layer) z-stack in order to demonstrate colocalization of red and green signals (GFP⁺ and cytokeratin⁺) and the absence of blue signal (CD45). No photostimulation or processing of artifact was performed. Statistics were performed using the *t*-test in all experiments.

Results

Autofluorescence experiments

Lung samples from mice exposed to all levels of radiation contained numerous bright green FITC⁺ cells by fluorescence microscopy. Samples from the 900-cGy TBI (experiment #1) that

were labeled with anti-GFP rabbit IgG Alexa Fluor 594 demonstrated that all FITC⁺ cells were also rhodamine⁺ and all rhodamine⁺ cells were also FITC⁺ (Fig. 1A, panels A–C). A three-dimensional image of one of these cells indicates colocalization of FITC and rhodamine signals (Fig. 1B). Lung samples from nonirradiated C57BL/6 mice or C57BL/6 mice irradiated with 900 cGy TBI and transplanted with C57BL/6 WBM did not have any FITC⁺ or rhodamine⁺ cells (Fig. 1A, panels D–F and G–I). These observations indicate that in irradiated wild-type mice transplanted with GFP⁺ WBM, FITC⁺ lung cells are truly GFP⁺ and not the result of artifact from autofluorescence as they label with anti-GFP antibody. Anti-GFP antibody only labels cells that are FITC⁺. No FITC⁺ cells are seen in the wild-type mouse lung and no cells nonspecifically label with anti-GFP antibody. In addition, radiation and transplantation with WBM do not appear to produce FITC⁺ cells or structures that could be considered GFP⁺. Appropriate isotype (rabbit IgG) and secondary antibody-only (anti-rabbit Texas red) controls for the anti-GFP antibody were performed and were negative. Thus, autofluorescence is not a problem under the conditions of these experiments.

Experiment 1: Bone marrow transplantation after radiation injury

Higher levels of donor cell bone marrow engraftment were seen with increasing doses of TBI. Radiation before marrow infusion was necessary to allow for engraftment as those exposed to no TBI had no detectable donor marrow–derived GFP⁺ cells in peripheral blood samples. The percent chimerism in those exposed to 500, 900, and 1200 cGy was 67.85 ± 6.46 , 87.62 ± 3.19 , and 94.14 ± 5.51 , respectively. The difference between each group was statistically significant ($p < 0.01$, all groups vs no TBI). With increasing doses of radiation, the lungs appeared more abnormal. One month after TBI, we noted that there was progressive alveolar septal thickening and hypercellularity with increasing doses of radiation (Fig. 2A, panels A–D) and increasing numbers of GFP⁺ cells within the lungs (Fig. 2A, panels E–H). More donor marrow–derived lung cells (GFP⁺ and CD45²) were seen in mice exposed to higher doses of radiation. It is important to emphasize that these cells represent nonhematopoietic pulmonary cells, which we have further specified by cytokeratin staining as outlined below. GFP⁺/CD45[−] cells in the lungs of mice exposed to 0, 500, 900, and 1200 cGy, expressed as a percentage of nucleated cells (DA-PI⁺) seen in 1003 high-power fields, was 0%, $1.17\% \pm 0.256\%$, $3.40\% \pm 0.938\%$, and $5.43\% \pm 0.355\%$. The difference between each group was statistically significant ($p < 0.01$) comparing all groups to each other) respectively (Fig. 2B,C). Additional immunohistochemical staining was performed on lung samples from the 500- and 900-cGy groups to further characterize the GFP⁺/CD45[−] cells. Many of these cells had the morphologic appearance and staining characteristics of alveolar epithelial cells, which were GFP⁺/CD45[−] and cytokeratin⁺. Cytokeratin staining of our lung samples yielded an anticipated pattern, labeling exclusively epithelial cells. Figures demonstrating this and the negative staining pattern of the isotype and secondary antibody-only controls are not included in this manuscript. In the 500-cGy group, 43.72% of all GFP⁺/CD45[−] cells were also cytokeratin⁺. This number increased to 61.43% in the 900-cGy group. The difference between both groups was statistically significant ($p = 0.05$). In Figures 3A and B, colocalization of GFP and cytokeratin without CD45 is demonstrated (panels A–D). A three-dimensional image of these cells further demonstrates colocalization of GFP and cytokeratin and not CD45 (Fig. 3A and B, panels E–G). We are showing redundant examples in Figure 3 because some in the field are asserting that the lung cell production from marrow cell phenomenon does not exist. Very rare GFP⁺/cytokeratin⁺/CD45⁺ cells were seen (Fig. 3A). These cells may represent cells in transition, which have gained cytokeratin but not yet lost CD45, or rare cytokeratin⁺ bone marrow cells. Most of the donor marrow–derived alveolar epithelial cells had the morphologic appearance of AE II; however, immunohistochemical labeling for AE II was not performed in this experiment. In a separate experiment, Mac-1 antibody labeling of lung samples demonstrated that Mac-1⁺ cells were universally CD45⁺, indicating that donor-derived lung cells were not macrophages.

Experiment 2: Bone marrow transplantation and mobilization with G-CSF after radiation injury

Mice in all groups achieved levels of peripheral blood chimerism of greater than 80%; however, there was no significant difference in chimerism between the four groups (Fig. 4). Radiation-injured mice that were mobilized with G-CSF were found to have more donor marrow-derived lung cells compared with nonmobilized, radiation-injured mice. GFP⁺/CD45⁺ cells in mice exposed to 900 cGy but no G-CSF, expressed as a percentage of nucleated cells (DAPI⁺), was $2.83\% \pm 0.44\%$. This percentage increased to $5.81\% \pm 0.56\%$, $7.88\% \pm 2.22\%$, and $5.74\% \pm 0.49\%$ in radiation-injured mice mobilized with one, two, and three rounds of G-CSF, respectively. There was no statistically significant difference in conversion when comparing the three mobilized groups ($p > 0.1$); however, statistical significance was achieved comparing the nonmobilized group to all three mobilized groups ($p < 0.01$).

Analysis in this experiment occurred at 9 weeks post TBI/BMT, whereas analysis in the radiation-injured mice from experiment #1 occurred at 4 weeks. There did not appear to be a significant difference in the percentage of GFP⁺/CD45⁺ cells in the 900-cGy TBI mice from experiment #1 (3.40%) compared with the 900-cGy TBI, no-mobilization mice from this experiment (2.83%, $p = 0.338$). Even 900-cGy TBI mice analyzed at 12 weeks from experiment #1 (3 mice) did not demonstrate a significantly different conversion rate compared with those analyzed at 4 or 9 weeks (3.04%, $p > 0.6$).

Experiment 3: Bone marrow transplantation after radiation and cardiotoxin injury

Although peripheral blood chimerism data was not available for all groups, the mean level achieved in the 900-cGy group was not significantly different from that of the 900-cGy group + cardiotoxin group ($p = 0.20$, Fig. 5). Cardiotoxin alone did not produce significant alterations in the appearance of the lungs by H & E staining 1 month after intramuscular injection and no GFP⁺/CD45⁺ cells could be seen in the lungs of mice given cardiotoxin alone; however, the number of these cells increased in mice given radiation and cardiotoxin compared with radiation alone. The difference between both groups was statistically significant (3.11 ± 0.762 vs 4.13 ± 1.08 , 900 cGy vs 900 cGy + cardiotoxin, $p = 0.05$). Data could not be collected from samples obtained in the 900-cGy-only group due to fixation problems with the lung tissue, so data from experiments #1 and #2 were used for comparison. Given the lack of a proper comparison group, these results should be interpreted with caution. Radiation-injured mice (900 cGy) that were given cardiotoxin and mobilized with two rounds of G-CSF achieved markedly higher levels of conversion in the lung. The percentage of all lung cells that were GFP⁺/CD45⁺ approached 19% in these two mice.

Experiment 4: Bone marrow transplantation with whole bone marrow and stem cell subpopulations after radiation and cardiotoxin injury

Peripheral blood chimerism in the Lin⁻, c-kit⁺, and Sca⁺ groups ($65.11\% \pm 4.67\%$, $67.11\% \pm 0.98\%$, and $64.65\% \pm 5.60\%$, respectively) was significantly higher compared with the Lin⁺, c-kit⁻, and Sca⁻ groups ($9.56\% \pm 2.12\%$, $2.82\% \pm 1.69\%$, and $1.06\% \pm 0.84\%$, respectively; $p < 0.01$). Chimerism in the Lin⁻, c-kit⁺, and Sca⁺ groups did not differ significantly compared with that of the WBM group ($50.52\% \pm 17.24\%$; $p > 0.2$; Fig. 6). A statistically significant increase in the number of donor cell-derived lung cells was also seen when Lin⁻, c-kit⁺, and Sca⁺ were transplanted ($1.81\% \pm 0.240\%$, $1.05\% \pm 0.109\%$, and $1.67\% \pm 0.259\%$, respectively) compared with Lin⁺, c-kit⁻, and Sca⁻ cells ($0.03\% \pm 0.045\%$, $0.02\% \pm 0.038\%$, and $0\% \pm 0\%$, respectively; $p < 0.01$). Conversion in the Lin⁻, c-kit⁺, and Sca⁺ groups did not differ significantly compared with conversion in the WBM group (1.47 ± 0.459 ; $p > 0.15$). Donor marrow-derived cytokeratin⁺ pulmonary epithelial cells were detected in the WBM, Lin⁻, c-kit⁺, and Sca⁺ groups, but not quantified.

Discussion

Bone marrow cells have been shown to be capable of producing pulmonary cells after in vivo transplantation. Reported conversion results and the degree of conversion have varied, as have the experimental design used in each study (Table 2). Kotton et al. [10] have reported the production of a small number (not quantified) of AE I from adherent marrow cells in the context of intratracheal bleomycin injury. In contrast, Theise et al. [11] have reported the production of AE II (14% of all AE II) from whole bone marrow or CD34⁺/Lin⁻ cells in the context of total-body irradiation. Using only one Fr25lin⁻ stem cell and helper whole bone marrow, Krause et al. [9] were able to demonstrate a high number of lung conversion products (up to 20% of all AE II) which were derived from the single transplanted stem cell.

There is ongoing controversy as to the identity of these donor-derived cells and the possible role of autofluorescence, as immunohistochemical labeling and cell imaging techniques have varied from study to study. Accordingly, we present data to show that autofluorescence is not a confounding variable. Concern has also been raised that erroneous results might be obtained because of cell overlap. By utilizing deconvolution microscopy and creating three-dimensional images of donor-derived cells, we can effectively demonstrate colocalization of GFP and cytokeratin (and not CD45) in the same cell and exclude the impact of overlapping cells. We are confident in the validity of our CD45 staining. We have found that nearly all marrow cells, with nucleated red blood cells being one exception, and all peripheral blood cells, stain with CD45. Very rare GFP⁺/cytokeratin⁺ cells that were also CD45⁺ were seen in our lung samples. These cells are of particular interest and may represent cells in transition, which have gained cytokeratin but not yet lost CD45, or rare cytokeratin⁺ bone marrow cells that have been previously described. Determining the identity of these cells is the subject of ongoing experiments. In addition, antibody labeling of paraffin-embedded lung samples with Mac-1 demonstrated that donor-derived lung cells were not macrophages as CD45⁻ cells were universally Mac-1⁻ (data not shown). Our data show that bone marrow cells are capable of producing pulmonary epithelial cells in vivo. In separate preliminary experiments, we have estimated that the majority of these pulmonary epithelial cells have the morphologic and phenotypic characteristics of AE II, as they label with prosurfactant protein B antibody. A smaller percentage have the morphologic and phenotypic characteristics of AE I, as they label with *Lycopersicon esculentum* lectin.

We have investigated the influence of many variables on conversion of hematopoietic marrow cells to lung cells and have demonstrated that the dose of irradiation and marrow mobilization with G-CSF both affect the degree of conversion in the lung. Cardiotoxin also injures the lung but augments the production of marrow-derived lung cells only marginally, demonstrating that variable outcomes accompany different modes of injury. We have also investigated the type of marrow cell responsible for the production of lung cells. Two candidates include hematopoietic stem cells and mesenchymal stem cells. Our data show that the cells possessing the phenotype of hematopoietic stem cells have the capacity to engraft marrow and produce lung cells. This does not exclude mesenchymal stem cells as participating in this phenomenon.

We demonstrated a clear radiation dose response for the production of lung cells from marrow cells. This could be due to direct lung damage and/or the establishment of marrow chimerism. Separate studies on the impact of radiation injury in the production of skeletal muscle cells from marrow cells indicate that cell production is primarily influenced by marrow chimerism rather than radiation-induced muscle injury. These issues need to be resolved. In this study, lung injury was clearly demonstrated in our histological correlates; however, the role of radiation-induced lung injury vs the establishment of marrow chimerism in the production of lung cells remains to be evaluated.

G-CSF administration, which mobilizes marrow-derived stem cells, also augmented lung cell production. These data suggest that increasing the number of circulating stem cells may impact the production of lung cells in the setting of lung injury; however, G-CSF has many actions and a direct effect on the lung, influencing lung cell production, cannot be excluded.

Fusion, as a mechanism for conversion, has been addressed in an increasing number of plasticity studies. Although it is an important issue, the presence or absence of fusion is model dependent and does not directly impact upon the significance of the findings from these studies. Harris et al. [26], using a Cre/lox recombinase transplant model, have indicated that marrow conversion to epithelial cells in the lung, liver, and skin may occur without cell fusion. Other models may show fusion.

In conclusion, these results indicate that significant levels of marrow conversion to lung cells can be routinely obtained by combining total-body irradiation with stem cell transplant followed by G-CSF administration. The administration of cardiotoxin as an additional lung injury had little impact on the production of lung cells. Other modes of cardiotoxin delivery and other injury modes must be further investigated. Certain hematopoietic cell populations (Lin^- , c-kit^+ , Sca^+) appear to preferentially engraft marrow and convert into lung cells, compared with other cell populations. These studies help to provide a base for investigating the use of stem cell transplantation as a treatment modality for various pulmonary disorders.

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Figure 1.

(A,B): GFP⁺ events are not due to autofluorescence. **A:** Lung samples from mice exposed to 900 cGy TBI were labeled with anti-GFP Alexafluor-594 (red) and DAPI nuclear counterstain (blue) and photographed using deconvolution fluorescence microscopy. There was red-green colocalization in all red or green cells observed in 10 random HPF from 5 samples (A: DAPI/FITC/rhodamine channels; B: FITC; C: rhodamine), indicating that all FITC⁺ cells were truly GFP⁺. Corresponding images from a nonirradiated C57BL/6 mouse (D,E,F) and a C57BL/6 mouse exposed to 900 cGy TBI and transplanted with C57BL/6 WBM (G,H,I) indicate that no cells or structures appear green or label with the anti-GFP Ab. **B:** Cells from a lung sample from the 900-cGy group labeled with anti-GFP Alexafluor-594, photographed using deconvolution fluorescence microscopy, demonstrates clear red-green colocalization, indicating that green cells are truly GFP⁺ (A: FITC/rhodamine channels; B: FITC; C: rhodamine). A three-dimensional view of one cell from the deconvolved image (asterisk) confirms colocalization (D,E,F).

Figure 2.

Radiation injury and the appearance of donor marrow–derived lung cells. **(A)**: Samples from radiation-injured mice 1 month after TBI demonstrating increasing cellularity and thickening of the alveolar walls by H & E staining (A–D: light) and more GFP⁺ cells (E–H: FITC/rhodamine filters) with escalating doses of TBI. No radiation (A,E); 500 cGy (B,F); 900 cGy (C,G); 1200 cGy (D,H). **(B,C)**: Results from the radiation injury experiment demonstrating that more donor marrow–derived lung cells (GFP⁺ and CD45[−]) were seen in mice exposed to higher doses of radiation. **(D)**: Samples from the radiation injury experiment were stained with anti-CD45 antibody using avidin Texas red as a secondary antibody and photographed using FITC (A), rhodamine (B), and FITC/rhodamine (C) filters. Native hematopoietic cells were considered to be CD45⁺ but GFP[−] (asterisk). Donor-derived hematopoietic cells were considered to be GFP⁺ and CD45⁺ (small arrow). Donor-derived lung cells were considered to be GFP⁺ and CD45[−] (large arrow).

Figure 3.

Donor-derived pulmonary epithelial cells.(A,B): Some of the donor-derived cells had the morphologic and phenotypic characteristics of pulmonary epithelial cells. Samples from all experiments were labeled with anti-CD45 (blue) and anti-cytokeratin (red) antibodies and photographed using deconvolution fluorescence microscopy. In A and B, the featured cells (arrow) are GFP⁺ (B: FITC filter), cytokeratin⁺ (C: rhodamine filter), and CD45⁻ (D: DAPI filter), indicating that they represent donor-derived pulmonary epithelial cells (A: all filters). A three-dimensional view of the featured cells from the deconvolved image confirms colocalization of GFP and cytokeratin, but not CD45 (E,F,G).

Figure 4.

Quantification of donor marrow-derived lung cells after radiation injury with G-CSF mobilization. **(A,B)**: Results from the radiation injury with G-CSF mobilization experiment demonstrating that more donor marrow-derived lung cells (GFP⁺ and CD45⁺) were seen in mobilized mice compared with nonmobilized mice.

B

cGy TBI	CTX	G-CSF	# mice	Average % blood chimerism (SD)	GFP+/CD45- cells as a % nucleated (DAPI+) lung cells (SD)
0	-	-	8	0 +/- 0	0 +/- 0
0	+	-	5	0 +/- 0	0 +/- 0
900 ¹	-	-	10	87.99 +/- 5.22	3.11 +/- 0.762*
900	+	-	8	81.70 +/- 12.09	4.13 +/- 1.08*
900	+	+	2	not available 18.9	(SD not done)

CTX = Cardiotoxin
¹Data from experiments #1,2
 *p = 0.05, GFP+/CD45- cells, 900 cGy vs. 900 cGy + CTX

Figure 5.

Quantification of donor marrow-derived lung cells after radiation and cardiotoxin injuries. (A,B): Results from the radiation and cardiotoxin injuries experiment demonstrating that more donor marrow-derived lung cells (GFP⁺ and CD45⁻) were seen in radiation and cardiotoxin-injured mice compared with radiation or cardiotoxin alone. When radiation and cardiotoxin-injured mice were mobilized with G-CSF, GFP⁺/CD45⁻ cells increased to 18.9% (2 mice).

Figure 6.

Quantification of donor marrow-derived lung cells after radiation and cardiotoxin injuries, using different donor cell subpopulation. **(A,B)**: Results from this experiment demonstrating higher levels of bone marrow engraftment and donor-derived lung cells (GFP⁺ and CD45⁺) in radiation and cardiotoxin-injured mice transplanted with donor cells possessing the phenotype of hematopoietic stem cells (Sca-1⁺, c-kit⁺, Lin⁻).

Table 1

Summary of experimental design

	Experiment 1	Experiment 2	Experiment 3	Experiment 4
Recipient mice	C57BL/6	C57BL/6	C57BL/6	C57BL/6
Donor mice	GFP ⁺	GFP ⁺	GFP ⁺	GFP ⁺
Donor marrow cells	Whole marrow	Whole marrow	Whole marrow	Sca ⁺ /Lin ⁺ , c-kit ⁺ /Lin ⁺ or WBM
TBI	0, 500, 900, or 1200 cGy	900 cGy	0, 500, or 900 cGy	500 cGy +
Cardiotoxin mobilization	[-]	[-]	[+/-]	[+]
G-CSF	[-]	0, 1, 2, or 3 rounds	0 or 2 rounds	[-]
Tissue analysis	4 weeks and 12 weeks post-TBI	9 weeks post-TBI	4 weeks post-TBI	4 weeks post-TBI

Table 2

Summary of selected lung conversion studies

[[Author]]	[[Injury]]	[[Donor cells]]	[[Conversion result]]
Krause et al. (2001) <i>Cell</i> 9	[[TBI]]	1 Fr25Lin ⁻ cell + WBM	Type II pneumocytes, 20.3% Bronchial epithelium, 3.74%
Kotton et al. (2001) <i>Development</i> 10	[[Intratracheal bleomycin]]	[[MSC]]	Type I pneumocytes (not quantified)
Theise et al. (2002) <i>Exp Hematol</i> 11	[[TBI]]	WBM or CD34 ⁺ Lin ⁻ cells	Type II pneumocytes, 14%
Jiang et al. (2002) <i>Nature</i> 22	[[TBI]]	[[MSC]]	[[Alveolar epithelium ~4%]]
Grove et al. (2002) <i>Am J Respir Cell Mol Biol</i> 12	[[TBI]]	[[WBM]]	Alveolar epithelium, 1–7% Type I and II pneumocytes
Abe et al. (2003) <i>Cytotherapy</i> 23	[[TBI]]	WBM or BM SP	Type I pneumocytes (not quantified) Bronchial epithelium (not quantified)
Ortiz et al. (2003) <i>PNAS</i> 24	[[Intratracheal bleomycin]]	MSC from bleo- resistant mice	Type II pneumocytes (not quantified)
Ishizawa et al. (2004) <i>FEBS Let.</i> 25	TBI + intratracheal elastase	[[Fetal liver cells]]	GFP ⁺ CD45 ⁻ cells (not identified) ~5% elastase/TBI only, ~17% elastase/TBI + ATRA, ~19% elastase/TBI + G-CSF, ~26% elastase/TBI + ATRA+ G-CSF Max GFP ⁺ CD45 ⁻ : 18.9%
Aliotta et al. (2005) <i>This study</i>	TBI + /-cardiotoxin	WBM, lin ⁻ /+, c-kit ⁻ /+, Sca-1 ⁻ /+	Type I, II pneumocytes, smooth muscle, endothelium

Key: TBI: total-body irradiation; cGy: centigray; NOD; Lin⁻: lineage-depleted; MSC: mesenchymal stem cells; WBM: whole bone marrow; BM SP: bone marrow side population cells; ATRA: *all-trans* retinoic acid; G-CSF: granulocyte colony-stimulating factor; Sca-1: stem cell antigen-1; Fr25: fractionated by elutriation.