Donor-Derived Type II Pneumocytes Are Rare in the Lungs of Allogeneic Hematopoietic Cell Transplant Recipients

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Abstract. Lung injury is a common cause of death and disability. Stem cell-related therapies are widely viewed as offering promise for people suffering from various types of pulmonary diseases, and gender-mismatched bone marrow transplant recipients serve as natural populations in which to study the role of bone marrow-derived stem cells in recovery from pulmonary injury. We evaluated the extent of lung repopulation by type II pneumocyte descendents of adult bone marrow-derived stem cells in allogeneic hematopoietic cell transplant recipients. Recut sections were obtained from five lung biopsy specimens and autopsy lung tissues from four female recipients of transplanted mobilized peripheral blood stem cells or bone marrow from male donors. Sequential immunohistochemistry and fluorescence in situ hybridization was performed on each section to evaluate for Y-chromosome-containing type II pneumocytes. A single Y-chromosome-containing type II pneumocyte was found in one lung biopsy from one hematopoietic cell transplant recipient. After adjustment for the effects of incomplete nuclear sampling, this pneumocyte represented 1.75% of all type II pneumocytes in the biopsy sample. There was no evidence of polyploidy to suggest cell-to-cell fusion. No donor-derived type II pneumocytes were found in samples from the other three patients. In conclusion, repopulation by bone marrow-derived stem cells or their progeny occurs at a low frequency in the lungs of hematopoietic cell transplant recipients. Conversely, proliferation by local stem cell populations appears to be more important for recovery from alveolar injury.

Keywords: stem cell, lung, type II pneumocyte, bone marrow, hematopoietic cell transplantation

Introduction

Lung injury is a common event in hematopoietic cell transplant patients owing to administration of high dose chemotherapeutic agents and radiation, increased susceptibility to infections, and graft-versus-host disease. Pulmonary injury can primarily involve the alveolar parenchyma, the conducting airways, or both. Diffuse alveolar damage is the most severe manifestation of lung injury and represents a common immediate cause of death in this patient group. The mechanisms leading to recovery are incompletely understood.

The participation of stem cells in alveolar recovery is a topic of current interest. Determination of the roles of resident and circulating populations of stem cells in adult life will be important for enabling progress towards augmenting the repair process. Over the years, research has supported a central role for resident type II pneumocytes in the recovery from alveolar injury [1-7], but the participation of circulating bone-marrow-derived cells and their progeny in the repair process has received less investigation. Clinical investigations and animal studies suggest that the lung can serve as a site of repopulation by bone-marrow-derived stem cells, but the results of these studies differ substantially. The current study was designed to investigate this question by using in situ hybridization techniques to evaluate lung biopsies from
gender-mismatched hematopoietic cell transplant recipients.

Materials and Methods

Institutional Review Board approval. This study was reviewed and approved by the University of Florida College of Medicine Institutional Review Board prior to its initiation, and was given an exemption from the requirement of written informed consent.

Patients. The University of Florida Shands Hospital Bone Marrow Transplant Program database, spanning the years from 1981 to 2001, was searched for female recipients of hematopoietic cell transplants from male donors. Four patients who met these criteria had transbronchial lung biopsy (TBB) or autopsy lung samples available (see Samples). All four patients entered into the study. Patients for the control groups were selected randomly from among the lung transplant recipients at the University of Florida. Three groups of patients were studied:

(A) Experimental Group: female recipients of transplanted mobilized peripheral blood (3 patients) or bone marrow (1 patient) from male donors (N = 4 patients/4 TBBS and 1 autopsy lung section)
(B) Male Controls: male recipients of transplanted lungs from male donors (N = 5 patients/5 TBBS)
(C) Female Controls: female recipients of transplanted lungs from female donors (N = 3 patients/3 TBBS).

Information about each patient’s age at transplantation and the indication for transplantation was collected for all subjects, and information about prior pregnancies was sought.

Tissue samples. For each subject, all lung tissue samples (biopsy specimens and autopsy materials) were included in the study. Recut sections were prepared from the archival tissue blocks that contained formalin-fixed paraffin-embedded lung tissues. Three slides from each TBB and one slide from the large autopsy tissue block were stained with hematoxylin-eosin, and an additional slide from each sample was stained with methenamine silver. One of the authors (DSZ) reviewed all of the slides to determine the pathologic diagnosis for each sample.

Immunohistochemistry. The methods have been previously described [8]. In brief, 4 µm thick recut sections from each experimental and control group tissue block underwent immunohistochemical staining followed by fluorescence in situ hybridization; a second slide from each block was prepared as a negative control. Sequential immunohistochemical staining was performed with monoclonal antibodies to human pro-surfactant protein B (SPB) (clone SPB01, titer 1:50, Research Diagnostics, Inc., Flanders, NJ) and human epithelial membrane antigen (EMA) (clone E29, titer 1:50, Dako Corp., Carpenteria, CA). For the anti-SPB staining, diaminobenzidine (DAB) was used as the chromogen, and for anti-EMA staining, Vector Blue was used as the chromogen. Counterstaining with hematoxylin or Nuclear Fast Red followed. SPB appeared as a finely granular brown material in the cytoplasm of type II pneumocytes, some Clara cells, and minimally in macrophages. Anti-EMA decorated the cell membranes of epithelial cells. Appropriate positive controls were run with each staining run, and internal controls present in all cases provided additional verification of the success of each staining run. Paired section-specific negative control slides from all cases were stained using an identical staining protocol, except for substitution of isotype-matched serum for each primary antibody, and demonstrated minimal or no non-specific staining. After the immunohistochemical staining was completed, all stained slides were comprehensively photographed using an Optronics digital camera (Optronics, Goleta, CA) attached to an Olympus BX51 microscope (Olympus America, Inc., Melville, NY) and a computer equipped with image capture and processing software.

Fluorescence in situ hybridization for X- and Y-chromosomes. These procedures were performed as previously described [8]. Probes included CEP X Spectrum Orange (alpha satellite) (Vysis, Inc., Downers Grove, IL), with specificity for the centromere region Xp11.1-q11.1 of chromosome X, and CEP Y Spectrum Green (satellite III) (Vysis, Inc.), with specificity for the DYZ1 locus contained within the Yq12 region of chromosome Y. Simultaneous use of in situ hybridization probes for both X- and Y-chromosomes permitted us to evaluate ploidy (and hence potential donor:recipient cell fusion) in all cases.

Evaluation of fluorescence in situ hybridization (FISH) preparations. All slides were comprehensively reviewed by one of the authors (DSZ) under an Olympus BX51 fluorescence microscope, which was equipped with a mercury lamp, a set of single-bandpass filters to detect DAPI, red fluorescence emissions above 585 nm, and green fluorescence emissions above 515 nm, and an Optronics digital camera, computer, and image capture and processing software. All type II pneumocytes demonstrating Y-signal were digitally photographed and the digital images stored for comparison with the photographs of the same immunohistochemically stained cells, to provide further assurance of the identity of the Y-containing cells as type II pneumocytes. X- (Cy-3) and Y-chromosome (FITC) signals appeared as dot-like or slightly elongated structures in the nuclei of cells. Cells with overlapping nuclei were excluded from analysis. Recognition of cells as type II pneumocytes was based upon morphologic characteristics (cuboidal cell morphology, finely vacuolated cytoplasm, round or oval nuclei, usually lying along an alveolar septum) and red-brown cytoplasmic fluorescence due to residual DAB, serving as a marker for SPB. Internal positive controls for the FISH (recipient and donor cells containing X- and Y-chromosomes) were visualized in every case, and slides from control group subjects were evaluated as follows: Percentages of type II pneumocytes containing Y-chromosomes were calculated for each of the Male Control Group slides, and then averaged to derive a correction factor to compensate for the incomplete nuclear sampling stemming from the use of 4 µm-thick sections. The
Table 1. Demographic, clinical, and histologic data.

<table>
<thead>
<tr>
<th>Subject – Sample #</th>
<th>Age at Tx</th>
<th>Indication for Tx</th>
<th>Graft source</th>
<th>Days after Tx</th>
<th>Pathologic diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 – 1</td>
<td>44</td>
<td>AML</td>
<td>PBSC</td>
<td>15</td>
<td>Diffuse alveolar damage</td>
</tr>
<tr>
<td>2 – 1</td>
<td>41</td>
<td>HD</td>
<td>PBSC</td>
<td>39</td>
<td>RT/chemo, previous hemorrhage</td>
</tr>
<tr>
<td>2 – 2</td>
<td></td>
<td></td>
<td></td>
<td>53</td>
<td>RT/chemo, previous hemorrhage</td>
</tr>
<tr>
<td>3 – 1</td>
<td>20</td>
<td>NHL</td>
<td>PBSC</td>
<td>23</td>
<td>Diffuse alveolar damage</td>
</tr>
<tr>
<td>4 – 1</td>
<td>54</td>
<td>CML</td>
<td>BM</td>
<td>314</td>
<td>Alveolar fibrin (early diffuse alveolar damage)</td>
</tr>
</tbody>
</table>

The sample labeled 2 - 2 contained one type II pneumocyte with a male gender karyotype. Abbreviations: Tx = transplantation, AML= acute myeloid leukemia, HD = Hodgkin’s disease, NHL = non-Hodgkin’s lymphoma, CML = chronic myeloid leukemia, PBSC = mobilized peripheral blood stem cells, BM = bone marrow, RT/chemo = radiation and chemotherapy effects,

3 Female Control Group slides were comprehensively inspected to evaluate for non-specific or cross-reactivity with the Y-chromosome probe, and showed no Y-signal.

**Calculation of the percentage of type II pneumocytes containing Y-chromosomes.** The number of type II pneumocytes was counted from the digital photographs for each sample with a Y-chromosome-containing type II pneumocyte, and the percentage of Y-signal-containing type II pneumocytes was calculated using the following formula: Percentage of Y-chromosome-containing type II pneumocytes = ([# Y+ type II pneumocytes / # type II pneumocytes in the sample] / mean fraction of Y+ type II pneumocytes in Male Control Group samples] x 100%

**Results**

Demographic, clinical, and histologic data for subjects are shown in Table 1. Subjects ranged in age from 20-54 yr. Patient 2 had borne a son, but patients 1, 3, and 4 had no known history of a pregnancy with a male child. Three of the 4 experimental group patients had received a transplant of mobilized peripheral blood stem cells, and only patient 4 received bone marrow. Time intervals between transplantation and biopsy or autopsy ranged from 15 to 314 days. The lung samples revealed either diffuse alveolar damage or radiation- and chemotherapy-associated changes accompanied by hemorrhage.

Sequential IHC and FISH highlighted one type II pneumocyte (Fig. 1) of male gender karyotype in the TBB from patient 2. This pneumocyte accounted for 1.75% of the 111 total type II pneumocytes in the sample, after correction for incomplete nuclear sampling. No other samples contained Y-signal-positive type II pneumocytes (Table 1). At least 100 pneumocytes were evaluated for each of these other samples. No XXX or XXXY cells, which would suggest fusion between recipient and donor cells, were identified in any sample.

**Discussion**

Our results indicate that the overwhelming majority of type II pneumocytes in the lungs of hematopoietic cell transplant recipients derive from the recipient’s own native lung. A pneumocyte of male gender was found in only one lung biopsy, accounting for one of 111 type II pneumocytes observed in this biopsy. Interestingly, this hematopoietic cell transplant recipient was the only patient in the experimental group who had borne a son. There have been previous reports of male karyotype cells in the lungs of women who have been pregnant with male fetuses, so we cannot exclude the possibility that the male gender karyotype pneumocyte in our patient’s lung is the result of microchimerism related to the male fetus of her earlier pregnancy [9,10]. Nonetheless, our results are similar to those we reported for gender-mismatched lung transplant recipients, in whom recipient gender karyotype type II pneumocytes were found in 5 of 7 subjects and 9 of 25 samples, and accounted for less than 1% of all type II
pneumocytes in the samples in which they were discovered [8].

Recent animal and human studies have suggested that bone-marrow-derived cells can differentiate into nonhematopoietic mature-appearing epithelial cells in the lungs, liver, gastrointestinal tract, skin, kidney, brain, and heart. The mechanisms that account for these phenomena are unclear, but possibilities include differentiation of nonhematopoietic precursor cells from the bone marrow, transdifferentiation of a cell from the bone marrow that had been previously committed to a different phenotype, and cell-to-cell fusion [11]. The frequencies of bone-marrow-derived epithelial cells in many organs appear to be quite low, on the order of one cell per thousand, or fewer [11].

Most animal studies have employed bone marrow transplantation models to evaluate pulmonary epithelial reconstitution via circulating bone marrow-derived cells. In these models, donor bone marrow expresses a marker (Y-chromosome, green fluorescent protein, lacZ, β-galactosidase) that enables distinction between cells derived from the donor and the recipient. The results of most of these investigations have supported the existence of pulmonary repopulation by bone marrow-derived cells. In a ground-breaking paper, Krause and colleagues [12] reported that a single bone marrow-derived stem cell injected into a mouse could produce progeny that could differentiate into bronchial epithelial cells and pneumocytes. After cross-gender bone marrow transplantation and lethal irradiation, Theise et al [13] found that up to 14% of the murine type II pneumocytes were derived from the donor. Similarly, Grove et al [14] reported that transgene-expressing cells accounted for up to 7% of cytokeratin-positive cells, with features of type II and type I pneumocytes, in lethally irradiated murine recipients of bone
marrow transplantation. Another study using transgenic mice further illustrated that pulmonary epithelial cells could develop from bone marrow-derived cells without cell-to-cell fusion [15]. We observed no evidence to support the presence of cell-to-cell fusion in our study.

In bone marrow transplanted mice treated with elastase to create emphysema, incorporation of donor-derived epithelial cells into the lung occurred in mice treated with all-trans retinoid acid or granulocyte colony stimulating factor [16], and was enhanced by administration of adrenomedullin [17]. Using a parabiotic mouse model and radiation and/or elastase to induce lung injury, investigators determined that cells with morphologic features of interstitial monocytes/macrophages, subepithelial fibroblast-like interstitial cells, and type I alveolar epithelial cells in one mouse were derived from the other mouse, presumably via circulating cells [18]. Lipopolysaccharide-induced airway injury in bone marrow transplanted mice was followed one week later by alveolar repopulation by donor-derived epithelial cells, at a mean rate of 15 per 200 alveoli [19]. Murine mesenchymal stem cells from the bone marrow administered systemically to minimally injured mice also generated donor-derived pulmonary epithelial cells [20].

In contrast, other animal studies that did not reveal evidence of pulmonary repopulation via circulating bone marrow-derived cells include those published by Wagers and colleagues [21], but their experimental methods differed substantially from previously published work, as discussed by Theise [22]. Kotton and colleagues [23] also did not find evidence to support bone marrow reconstitution of pulmonary epithelial cells.

Investigations of human bone marrow transplant recipients have yielded varying results regarding the existence of pulmonary epithelial chimerism. Suratt and colleagues [24] studied lung samples from 3 female cross-gender hematopoietic cell transplant recipients and reported 2.5 – 8.0% epithelial chimerism in 2 of the 3 patients' lungs. Donor-derived epithelial cells were described as lying primarily in alveolar locations, and occasionally in bronchioles. A similar frequency of chimeric epithelial cells was reported in another study of 2 female cross-gender hematopoietic cell transplant recipients [25]. Conversely, Kleeberger et al [26] found no epithelial chimerism in bronchial tissue samples from 3 bone marrow transplant recipients, but pneumocytes were not available for analysis.

Our results confirm the presence of pulmonary epithelial microchimerism in one hematopoietic cell transplant recipient, but the frequency of donor-derived pneumocytes we observed was lower than frequencies previously reported. This may be related to differences in the experimental methods, particularly in the number of pneumocytes evaluated in each sample and the degree of stringency about the decision whether to exclude individual cells from consideration based upon overlapping of their nuclei. We were very stringent about excluding cells with overlapping nuclei, and so may have underestimated the number of donor-derived pneumocytes. On the other hand, a less stringent approach may falsely elevate the number of donor-derived pneumocytes. Other potential explanations for the differences in our results include differences in tissue preservation, in the nature and longevity of the lung injury at the time the samples were obtained, and in the degree of engraftment.

Nonetheless, the results of the earlier and current studies indicate a dominant role for resident stem cells in alveolar repair and regeneration. The very small numbers of donor-derived pneumocytes observed are unlikely to contribute significantly to these processes. Although substantial work has been done in the last few years to elucidate the identities of stem cells in the lung, much remains to be learned about the functions of these cells, and potential approaches to assisting alveolar recovery. These processes represent an important focus for future investigations.

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