Retention of human bone marrow-derived cells in murine lungs following bleomycin-induced lung injury

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Submitted 4 June 2007; accepted in final form 23 May 2008

Although most lung injury is repaired by locally derived progenitor cells, recent information suggests that cells that originate outside the injured organ, presumably derived from bone marrow, may also repopulate the lung. Using tissue from human lung or bone marrow transplant recipients, investigators have identified cells in the lung that express different genetic profiles than those of the tissue's source, implying that some cells were not lung derived. For example, in female patients who had undergone sex-mismatched bone marrow transplants, 2–8% of lung epithelial cells were found to express donor-derived Y-chromosome-positive cells, suggesting bone marrow origin of these cells (36, 46). In another study, following lung transplantation, up to 24% of lung epithelial cells were found to be recipient derived, most notably in areas of chronic injury (22). Using animal models of lung injury, investigators localized exogenously derived cells to the lung following either transplantation with labeled bone marrow (2, 19, 50) or direct infusion of bone marrow-derived cells (BMDC) (27, 38).

Given these findings, there has been increasing interest in administering BMDC, either to repair an injured lung or to deliver therapeutic agents to enhance repair or correct a known defect, as in cystic fibrosis (12, 34, 49). However, when adult BMDC have been administered intravenously as a single dose to animals (usually mice) with experimental lung injury, results have been mixed, often with little or no engraftment seen (6, 27, 38). Whole bone marrow contains many different cell types, including hematopoietic and non-hematopoietic stem cells, which have demonstrated the ability to differentiate into mature cells of a variety of tissues including lung (16). However, there is concern that some bone marrow-derived progenitor cells may express a fibrocyte phenotype, contributing to a fibrotic response, rather than to a reconstitution of normal lung architecture (13, 40). The capacity for engraftment of human BMDC, in contrast to murine BMDC, in injured lung has not been previously investigated. It is important to determine the fate of human BMDC in the lung before therapeutic uses of these cells for human lung diseases is attempted, since human cells may behave differently than murine cells under similar conditions (9).

Circulating progenitor cells are directed to the bone marrow, and potentially other organs, by chemotactic signals. Stromal-derived factor-1 (SDF-1; also called CXCL12), along with its receptor, CXCR4, appear to be the most important peptides determining homing and engraftment of BMDC (32, 44). SDF-1 is a member of the CXC chemokine family and is highly conserved among species, including human and mouse (31). Homing and retention of stem cells into injured organs may also be directed by the relationship between SDF-1 and CXCR4, suggesting that modulation of these interactions could enhance stem cell engraftment following injury (3, 20, 25). Christopherson et al. (7) found that endogenous expression of CD26 (dipeptidylpeptidase IV) on donor cells negatively influences SDF-1/CXCR4 interactions. They reported that pre-incubation of mouse hematopoietic stem cells with Diprotin A (Ile-Pro-Ile), a reversible inhibitor of CD26, resulted in increased homing and engraftment of the infused stem cells.

In this study, we used well-characterized mouse xenograft models to determine the potential of adult human BMDC to be retained in the lungs of immunodeficient mice following a single intravenous infusion. Since previous reports suggested the need for lung injury to be present to show significant levels...
of BMDC engraftment, we studied mice with and without bleomycin (bleo)-induced lung injury. We also investigated whether preincubation with Diprotin A could enhance retention in lung of human BMDC.

MATERIALS AND METHODS

Animals. Nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice and NOD/SCID/β2 microglobulin-deficient (β2Mnull) mice were purchased from Jackson Laboratory (Bar Harbor, ME). NOD/SCID/β2Mnull mice have reduced natural killer cell activity and have demonstrated increased human cell engraftment compared with NOD/SCID mice in other studies (23, 37). Animals were raised in a pathogen-free environment in filtered cages and given filtered, acidified water and irradiated food ad libitum to minimize infection. All animal experiments were approved and performed according to the Institutional Animal Care and Use Committee guidelines of the University of Southern California Keck School of Medicine.

Bleo-induced lung injury. Bleo (Sigma, St. Louis, MO; 4 U/kg in NOD/SCID mice or 2 U/kg in NOD/SCID/β2Mnull mice) or saline was administered in 40- to 50-μl aliquots intranasally to mice 4–6 wk old after sedation with ketamine/xylazine. Intranasal administration of bleo to mice has previously been found to be as effective as intratracheal administration in producing bleo-induced lung injury (45). Doses of bleo were individualized to each mouse strain and were based on induction of visible lung injury with low lethality. Representative mice were killed for histological analysis of lung tissue by fixation with 4% paraformaldehyde and paraffin embedding. To confirm the development of an acute lung injury that evolves to fibrosis, lung tissue slides were exposed to a trichrome stain, which stains collagen blue.

Immunofluorescence. Slides from NOD/SCID/β2Mnull mouse lung tissue were labeled using an anti-SDF-1 antibody (Ab). This mouse monoclonal anti-SDF-1 Ab (R&D Systems, Minneapolis, MN) was developed against a peptide from human SDF-1α, and, according to the manufacturer, cross reacts with both mouse and human SDF-1α and β. Mouse IgG1 (Sigma) was used as control. Following deparaffinization and microwave antigen retrieval (Antigen Unmasking Solution, Vector Laboratories, Burlingame, CA), slides were incubated with mouse SDF-1 Ab or mouse IgG1 control Ab. SDF-1 signal was amplified using the Catalyzed Signal Amplification kit (CSA; Dako), substituting Cy3-strepavidin (Jackson ImmunoResearch Laboratories, West Grove, PA) for the chromogen. Slides were treated with Vectashield (Vector) containing 4',6-diamidino-2-phenylindole (DAPI), which counterstained nuclei blue. Slides were viewed using a Nikon Eclipse 80i microscope with epifluorescence optics. Images were captured separately using a cooled charge-coupled device camera (QImaging, Surrey, BC, Canada), merged, and imported into Adobe Photoshop (Adobe Systems, Mountain View, CA) as TIFF files.

Human BMDC preparation and administration. Whole adult human bone marrow cells (Cambrex, Walkersville, MD or AllCells, Emeryville, CA) were used as the source of BMDC under a protocol approved by the Institutional Review Board of the University of Southern California. Each set of experiments was performed with whole bone marrow aspirates from different donors. Whole bone marrow cells were prepared using a Ficoll gradient and then labeled with CellTracker Green CMFDA (5-chloromethylfluorescein diacetate; Molecular Probes, Eugene, OR). To determine the optimal time of BMDC injection in relation to bleo injury, 5 × 105 CMFDA-labeled BMDC were washed, resuspended in Hanks’ buffer with heparin (50 U/ml), and administered via tail vein injection to NOD/SCID or NOD/SCID/β2Mnull mice 1, 2, 3, or 4 days post-bleo. In select experiments, cells were preincubated with 5 mM Diprotin A (Sigma) for 15 min at 37°C, washed, and similarly resuspended in Hanks’ buffer with heparin before tail vein injection.

Flow cytometry analysis. Mice were killed post-bleo injury day 7. Lungs were removed and enzymatically digested using dispase (BD Biosciences, San Jose, CA) and Blendzyme (Roche, Indianapolis, IN), yielding a mixed population of lung cells. Cells were passed through 100-, 40-, and 20-μm filters to obtain single cell suspensions and analyzed by flow cytometry using the FACSCalibur machine (BD Biosciences). CMFDA+ cells were gated in the green fluorescent channel, counted, and recorded as % of nucleated cells. In selected experiments, cells were labeled with anti-mouse or anti-human CD45 antibodies, or isotype-specific control Ab, conjugated to allophycocyanin (APC; BD Biosciences).

Statistical analyses. Data are expressed as means ± SE. For comparisons of multiple time points and conditions, two-way ANOVA was performed, followed by post hoc procedures based on modified Newman-Keuls tests using the statistical program GB-STAT v.9 (Dynamic Microsystems, Silver Spring, MD). P < 0.05 was considered to be statistically significant.

RESULTS

Bleo-induced lung injury in immunodeficient mice. Intranasal administration of bleo to NOD/SCID or NOD/SCID/β2Mnull mice resulted in visible lung injury and weight loss of ~10–20% at day 4, with mortality <5%. We sought to confirm the development of acute lung injury followed by fibrogenesis and the laying down of collagen by histological examination of NOD/SCID/β2Mnull mice. Using trichrome staining, we found no collagen deposition in the distal lung parenchyma of control lungs (Fig. 1, A–C). At day 4, inflammation dominated the findings in the injured areas in bleo-treated animals, with the beginnings of collagen deposition already apparent (Fig. 1, D and E). By animal death on day 7, collagen was seen throughout the injured areas (Fig. 1, F and G).

Localization of human BMDC to mouse lung. BMDC labeled with CMFDA showed >95% viability and were easily detected in the green channel of the flow cytometer. To determine the degree of labeling and short-term stability of the label, aliquots of unlabeled BMDC and CMFDA-labeled cells were saved for up to 4 days at 4°C in 90% FBS and analyzed by flow cytometry (Fig. 2). Greater than 95% of BMDC were found to still express the CMFDA label. Thus, nearly all BMDC, regardless of their cell type, took up the CMFDA label and continued to express the label for at least 4 days. To learn whether the label was stable over a longer period of time and through several cell divisions, cultured human mesenchymal stem cells (MSC) labeled with CMFDA were used as control (since all cellular components of whole bone marrow cannot be reliably cultured). We found the CMFDA label to persist in cells for at least 4 wk in culture through multiple passages (data not shown). To determine whether CMFDA+ cells could be detected by flow cytometry within a mixed population of isolated lung cells after a single intravenous infusion, we infused CMFDA-labeled MSC to uninjured mice and killed animals within 2 h of infusion. Bright green cells could be readily identified, likely representing many cells that were entrapped in lung capillaries at that early time point (data not shown) and demonstrating the feasibility of this approach.

Since lung injury and repair is a dynamic process, we were interested in learning which day might offer the best chance for retention of donor BMDC within the first few days after injury. In a pilot study of 1–3 mice for each condition, we infused CMFDA-labeled BMDC by tail vein injection into NOD/SCID mice at days 1–4 after saline or bleo treatment. When lung cells were enzymatically dispersed and analyzed by flow cytometry at day 7 after bleo, minimal numbers of CMFDA+...
cells were found, representing <0.005% of the total cells analyzed at any time point. We then studied NOD/SCID/β2Mnull mice, which have produced higher levels of human cell engraftment in other experimental models (23) to see whether improved retention could be achieved. There was a 10- to 100-fold increase in the percentage of CMFDA+ cells seen in NOD/SCID/β2Mnull mice compared with NOD/SCID mice, with ∼0.04% positive cells seen when cells were infused at day 4 and mice were killed at day 7 after bleo (Fig. 3). There were few CMFDA+ cells in saline control lungs. In these pilot experiments, both NOD/SCID and NOD/SCID/β2Mnull mice showed a trend toward increased retention of BMDC when cells were infused 4 days after bleo (albeit at low levels), thus, NOD/SCID/β2Mnull mice were administered CMFDA-labeled BMDC at day 4 after bleo or saline and killed at day 7 after bleo for all subsequent experiments.

There was a significant increase in the percentage of CMFDA+ cells seen in NOD/SCID/β2Mnull mice compared with NOD/SCID mice, with ∼0.04% positive cells seen when cells were infused at day 4 and mice were killed at day 7 after bleo (Fig. 3). There were few CMFDA+ cells in saline control lungs. In these pilot experiments, both NOD/SCID and NOD/SCID/β2Mnull mice showed a trend toward increased retention of BMDC when cells were infused 4 days after bleo (albeit at low levels), thus, NOD/SCID/β2Mnull mice were administered CMFDA-labeled BMDC at day 4 after bleo or saline and killed at day 7 after bleo for all subsequent experiments.

There was a significant increase in the percentage of CMFDA+ cells in lungs of all bleo-injured NOD/SCID/β2Mnull mice at day 4 (0.040% ± 0.006%, n = 20) compared with all saline controls (0.009% ± 0.003%, n = 7). However, donor cells still represented an exceedingly small percentage of cells in the lung. In an attempt to increase retention of human BMDC in bleo-injured mouse lung, we investigated whether enhancement of the SDF-1/CXCR4 axis would increase the trafficking of human cells to the injured lung. We localized SDF-1 to the bleo-injured lung by staining paraformaldehyde-fixed and paraffin-embedded lung tissue from NOD/SCID/β2Mnull mice at
day 4 after injury, the same day that retention was greatest as noted by flow cytometry. Small amounts of SDF-1 were found scattered throughout the lung parenchyma in a representative saline control animal (Fig. 4A). In contrast, in a representative bleo-injured mouse lung, abundant SDF-1 protein was present in affected areas (Fig. 4B). There was no staining using an isotype-specific control Ab instead of the SDF-1 Ab (Fig. 4C).

After finding SDF-1 increased in bleo-injured lung tissue, we sought to augment the chemoattractant effect of SDF-1 by incubating BMDC in Diprotin A before infusion. Representative flow cytometry results are shown in Fig. 5. Human hematopoietic cells, as determined by anti-human CD45 Ab labeling, accounted for >90% of CMFDA+ cells retained at bleo or saline day 7 and was not different between saline- or bleo-treated mice. To learn whether or not the CMFDA+ cells were actually mouse macrophages that had ingested the CMFDA-labeled BMDC, we analyzed aliquots of cells by incubating with an anti-mouse CD45 Ab conjugated to APC. The CMFDA+ cells did not colocalize with mouse CD45, suggesting that the uptake of BMDC by phagocytes was likely not an important component of the CMFDA+ population (data not shown). Preincubation of BMDC with Diprotin A resulted in a modest increase (~30%) in the percentage of CMFDA+ cells retained in the lungs of bleo-treated mice (n = 10, Bd4 mice given BMDC with preincubation with Diprotin A, n = 10, Bd4 given BMDC preincubated in vehicle only), as determined by flow cytometry, which did not achieve statistical significance (Fig. 6).

**DISCUSSION**

The ability of BMDC to engraft in organs remote from bone marrow suggests that exogenously administered cells may contribute to the repair of the pulmonary alveolar epithelium following injury. This could be of enormous clinical benefit for regeneration of injured lung tissue. However, key questions remain unanswered, including the optimal types of cells to be used, the most favorable timing of cell infusion, the best route of administration, and whether engraftment or differentiation requires enhancement of recruitment before or during the process of transplantation. Most previous studies have evaluated the contribution of rodent BMDC to lung repair, but it is unclear whether human BMDC are capable of responding in a similar fashion. We attempted to address some of these questions using human BMDC in experimental lung injury using an immunodeficient mouse xenograft model. We chose to study...
Retention of BMDC in bleo-injured lung tissue, reasoning that engraftment of the infused cells would be unlikely without cells being available in the lung for at least the first several days after injury.

In this study, we evaluated if, after a single intravenous infusion, adult human CMFDA-labeled BMDC could be identified in the lungs of immunodeficient mice following experimental lung injury. Retention was minimal using NOD/SCID mice and greater using more immunodeficient NOD/SCID-2Mnull mice. Other investigators have similarly found NOD/SCID-2Mnull mice, which lack natural killer cell activity, to be superior recipients of human stem cell xenografts (23, 37). Few BMDC were seen in saline control animals, supporting previous observations that tissue injury is required for exogenous stem cell engraftment (18). Timing of infusion of BMDC after injury may have had an important effect on retention of CMFDA+ cells in the lung, with the highest percentage seen when cells were infused 4 days after bleo injury. Of note, the infusion of BMDC at day 4 after bleo, as well as being the point of maximal cell retention, was also the shortest time period between infusion and analysis and which may confound interpretation of the results with regard to optimal timing of infusion following bleo. However, the main goal of this study was to assess overall retention of human BMDC in a murine xenograft model rather than to evaluate and make definitive conclusions regarding the optimal time of infusion. It is possible that some BMDC had been trapped in injured lung tissue and perhaps cleared at a later time point. However, even under these favorable conditions, CMFDA+ cells were still found to represent a very small percentage of total lung cells, as measured in isolated lung cells by flow cytometry, at ~0.04% at the peak time point. There was ~30% increase in CMFDA+ cells detected when BMDC were preincubated with Diprotin A. Nearly all (>90%) of the CMFDA+ cells found in lung expressed human CD45+. We conclude that human BMDC are retained in the lung following injury, albeit in low numbers in this mouse xenograft model. However, nearly all of the cells expressed a hematopoietic cell marker, suggesting that the retained BMDC did not differentiate into lung parenchymal cells, at least at this early time point after injury.

Intratracheal bleo results in a well-characterized injury in immunocompetent animals that evolves from an early inflammatory stage to later fibrosis (43). Immunodeficient SCID mice have previously been administered intratracheal bleo with resultant lung injury (14, 40). However, it was unknown whether the more severely immunodeficient NOD/SCID or NOD/SCID-2Mnull mice would be able to mount a sufficient inflammatory response and demonstrate similar pathological changes without significant mortality. By using a transnasal approach and titrating the appropriate dose of bleo, we consistently observed considerable levels of injury, confirming this to be a useful model with which to investigate recruitment of BMDC to lung following injury.
Types of adult BMDC that may optimally contribute to lung engraftment are as yet unknown. Since the most vigorous engraftment has been seen with cells following bone marrow transplantation (2, 18), we infused whole BMDC to look for possible retention. In addition to differentiated hematopoietic and other cell types, several different progenitor cell populations are expected to be represented in this cell mixture, including hematopoietic stem cells (26), MSC (5), multipotent adult progenitor cells (21), and progenitors for endothelial cells (11), epithelial cells (10), fibrocytes (30, 40), and possibly others. Some of these cells are believed to retain remarkable plasticity, in that they can differentiate into cells of endodermal, ectodermal, or mesodermal origin (28, 41). It is unclear whether infusion of subsets of cells from bone marrow would preferentially engraft in distant organs, compared with stem cells infused as part of a whole marrow population (1). Although exogenously derived stem cells are thought to differentiate into these various cell types, it is possible that some stem cells may engraft by fusing with existing cells (17). In any event, our study suggests that a single infusion of whole BMDC may not be sufficient to establish sufficiently useful engraftment into injured lung tissue.

Circulating stem cells are likely recruited to an injured lung by chemotactic signals, similar to bone marrow transplantation (32). In a bone marrow transplant xenograft model using NOD/SCID mice and human stem cells, stem cell engraftment has been found to be dependent on the chemokine SDF-1 and its receptor CXCR4 (33, 39). The cellular expression of CXCR4 is likely variable and at least partially dependent on local conditions. For example, using human cord and fetal blood-derived stem cells, Kollet et al. (24) found that even CXCR4-negative cells migrated to a SDF-1 gradient following cytokine stimulation, as may be present in injured lung tissue. This response was mediated by upregulation of both surface and cytoplasmic CXCR4. Other investigators have shown that exposure to growth factors (35) or to hypoxia (42) also enhance CXCR4 expression and increased chemotactic response to SDF1. Conditions in bleo-injured lung tissue are thus likely conducive to expression of CXCR4 in infused BMDC. SDF-1 is expressed by bone marrow endothelial and stromal cells, with human cells capable of responding to mouse-derived SDF-1. Epithelial cells in many other organs also express SDF-1, suggesting that it may be important in the recruitment of circulating cells that express CXCR4, such as BMDC (29). We demonstrated that SDF-1 was indeed present in injured areas by immunofluorescence labeling of whole lung tissue. This corresponds to a previous report that SDF-1 rises in whole lung lysates after bleo lung injury, peaking between days 2–16 after bleo (40). To improve stem cell engraftment, various investigators have attempted to enhance SDF-1/CXCR4 interactions. CD26 is a membrane-associated peptidase that selectively cleaves dipeptides with a proline or alanine in the second position at the NH₂ terminus, such as occurs with SDF-1 (8). This negatively alters the cellular response to SDF-1 and reduces homing and engraftment of CXCR4+ cells (7). By preincubating BMDC with Diprotin A, a reversible inhibitor of CD26, we were able to modestly increase the percentage of retained cells, although the results did not achieve statistical significance.

Recent investigations have raised the possibility that administration of stem cells to injured tissue may have therapeutic effects distinct from those related to actual engraftment, such as in monocrotaline-induced pulmonary hypertension (4), ischemic acute renal failure (47), and myocardial ischemia (48). It has been proposed that these beneficial responses may be mediated by complex paracrine responses that may support tissue repair without a persistent presence in the target tissue (15, 47, 48). Although retention of stem cells could potentially be beneficial in this context in the absence of engraftment, the relative contribution of other mechanisms to improve tissue regeneration remains to be fully elucidated.

In summary, we administered CMFDA-labeled human BMDC to immunodeficient mice by tail vein injection after bleo-induced lung injury to learn whether those cells would be retained in the lung for possible engraftment. We found a very small percentage of human cells in the lung in NOD/SCID mice, with a modest increase in retained cells detected in lungs of NOD/SCID/β2-Mnull mice. The timing of infusion of BMDC after injury appeared to have an effect on the percentage of cells later detected in the lung, with the highest percentage of cells retained in the lung 4 days after bleo injury, although retention may also be influenced by the day of analysis after BMDC infusion. Preincubation of cells with Diprotin A slightly increased the percentage of retained cells. The majority of retained cells were human CD45+ , suggesting that the potential for differentiation to a non-hematopoietic phenotype using BMDC is limited, at least at these early time points following injury. Given the overall low number of human cells detected in injured lung, additional methods to increase homing and retention of adult BMDC, and consideration of other stem cell populations, will likely be required to facilitate engraftment in the treatment of lung injury.

ACKNOWLEDGMENTS

The authors note with appreciation the statistical assistance of Nazanin Yacobi and Kwang-Jin Kim.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Research Grants HL-038578, HL-038621, HL-062569, and HL-072231 and by the Hastings Foundation. E. D. Crandall is Hastings Professor and Kenneth T. Norris Jr. Chair of Medicine.

REFERENCES

HUMAN BMDC FOLLOWING LUNG INJURY

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