Bone Marrow–derived Lung Epithelial Cells

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Bone marrow–derived cells can take on the phenotype of epithelial cells and express epithelial-specific genes in multiple organs. Here, we focus on recent data on the appearance of marrow-derived epithelial cells in the adult lung. These findings have garnered significant skepticism because in most cases marrow-derived epithelial cells are very rare, the marrow cell of origin is not known, the techniques for detection have needed improvement, and there seem to be multiple mechanisms by which this occurs. Recent studies have focused on these concerns. Once these important concerns are addressed, further studies on the function(s) of these cells will need to be performed to determine whether this engraftment has any clinical significance—either beneficial or detrimental.

Keywords: stem cell plasticity; surfactant protein C; cell fusion

DEMONSTRATIONS OF MARROW-DERIVED EPITHELIAL CELLS

Several studies suggest that bone marrow (BM) cells may promote repair of injured pulmonary epithelium (1–6). In 2001, Kotton and colleagues demonstrated that marrow stromal cells had the ability to engraft as rare pneumocytes after intravenous infusion into mice that had undergone lung injury with bleomycin (4). The number of marrow-derived epithelial cells was significantly higher in animals injured with bleomycin compared with control mice that did not undergo tissue damage. Another representative study is that performed by Abe and coworkers, who compared different BM-derived populations for their ability to differentiate into lung (and liver) epithelial cells in mice that were either uninjured or had undergone whole body irradiation, which causes transient acute lung injury (7). The donor mice used expressed enhanced green fluorescent protein (GFP). Engraftment of rare bone marrow–derived side population (SP) cells was compared with that of whole bone marrow. (SP cells are so named because their fluorescence emission after exposure to Hoechst dye is lower, and to the side of, most cells in the marrow due to expression of transporters that actively extrude the dye.) Within the bone marrow SP cell population, there are hematopoietic stem and progenitor cells, as well as nonhematopoietic cells whose identity is not yet well defined. Two important findings were reported. First, they showed that marrow-derived epithelial cells were only found in mice that had undergone radiation-induced lung injury. Second, after radiation-induced injury; the SP cell population was enriched for cells that engrafted as rare type I pneumocytes and airway epithelial cells. Thus, lung injury is required for the appearance of marrow-derived epithelial cells, and bone marrow cells within the SP population are enriched for cells capable of taking on the phenotype of lung epithelial cells.

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My laboratory has similarly shown engraftment of lung epithelial cells after transplantation of whole bone marrow into lethally irradiated mice. We used the Y chromosome as a marker of marrow cell origin. Any cell derived from the engrafted population, by any mechanism (fusion or differentiation), would contain a Y chromosome. In our experience, we were unable to definitively identify type I pneumocytes, but we were able to detect low levels (0.1%) of type II pneumocytes that were marrow derived. In addition, my laboratory has determined that there is a threshold of lung injury required for the appearance of marrow-derived epithelial cells in the lung (8). With irradiation doses less than 800 centigray, no marrow-derived type II pneumocytes were identified. In contrast, at doses of greater than 1,000 centigray, we identified marrow-derived type II pneumocytes in at least 50 percent of mice. It was exceedingly rare for us to detect bone marrow–derived epithelial cells lining the airways; with perhaps one to two cells definitively identified in the airways of 30 to 40 transplant recipients that had undergone high dose irradiation.

THESE FINDINGS HAVE GENERATED CONTROVERSY

Despite several manuscripts that definitively show marrow-derived epithelial cells, these data are still very controversial. The primary concerns have been (*1*) whether the findings themselves are actually detection artifacts and thus do not represent true bone marrow–derived epithelial cells, (*2*) that the frequency of bone marrow–derived epithelial cells is too low to be physiologically relevant, and (*3*) that the primary mechanism responsible for the appearance of bone marrow–derived epithelial cells is cell fusion. Significant efforts are required to address each of these legitimate concerns. Below, I highlight efforts in my lab and those of others that relate to these controversies.

TECHNICAL CONSIDERATIONS FOR DETECTION OF BM-DERIVED EPITHELIAL CELLS

Need for Definitive Approaches for Identifying Marrow-derived Epithelial Cells

There are many technical challenges in the detection of bone marrow–derived epithelial cells. As this field has evolved over the last decade, certain endpoints have been determined to be essential for data to be definitive. The most important aspects are (*1*) proving that a cell is donor derived by the use of markers such as the Y chromosome or a donor-specific gene (either endogenous or a transgene), (*2*) proving that a cell is truly epithelial by the expression of cell specific markers, and (*3*) ruling out overlay of cells. Alternate/complementary approaches include use of a reporter system in which expression of a gene is specific for the epithelial cell type of interest and also for the donor cells. For example, we have transplanted wild-type bone marrow cells into surfactant protein C (SP-C)–null recipient mice (9). In this system, the only cells that express SP-C are type II pneumocytes in the lung, and the recipients completely lack this gene. Therefore, any SP-C+ cells identified after transplantation of wild-type bone marrow into SP-C–null recipients must be expressing the SP-C gene provided by the

donor cells. Another example for definitive identification of marrow-derived epithelial cells is to isolate single cells for analysis of donor cell markers and epithelial-specific gene expression by immunohistochemistry, fluorescence-activated cell sorting (FACS), or culture characteristics.

Tissue Preparation

For thorough analysis of lung tissue for BM-derived epithelial cells, multiple experimental approaches could and should be taken. First, at the time of killing, right heart perfusion should be performed to wash out intravascular blood cells from the lung bed. Lung tissue should then be obtained for paraffin sections as well as for enzyme-mediated (e.g., dispase) digestion for analysis of single cells for flow cytometric, *in vitro* culture and/or cytospin analysis. The degree of fixation is critical for most detection approaches. For example, to perform both Y chromosome fluorescence *in situ* hybridization and immunofluorescence on the same paraffin-embedded tissues, it is important that the tissues not have been overly fixed in paraformaldehyde. We generally make small pieces of tissue and fix them in buffered paraformaldehyde for just 2 to 4 hours.

Immunofluorescence for a Nuclear Protein Plus Y Chromosome FISH

We have performed studies comparing and contrasting different methods for identifying marrow-derived epithelial cells. In the course of these experiments, not only have we developed better, more definitive techniques for identifying these cells, but we have also discovered that different strains of transgenic mice that presumably express green fluorescent protein ubiquitously, and which are often used to track marrow-derived cells, have widely variant levels of expression of GFP (10). We have established an improved method for the detection of marrow-derived hepatocytes (11), and similar methods are also being applied for detection of marrow-derived epithelial cells in the lung. When one is using Y chromosome fluorescence *in situ* hybridization (FISH) for detection of marrow-derived cells, the Y chromosome signal is, of course, in the nucleus. It has been difficult to definitely prove that a Y+ nucleus is surrounded by a specific cytokeratin+ cytoplasm due to potential overlay in solid organ sections. Therefore, if we could use immunofluorescence for a nuclear protein that is specific to a particular epithelial cell phenotype and completely absent from hematopoietic cells, this would provide a more rigorous approach for identifying marrow-derived epithelial cells in tissue sections by nuclear staining. For example, in the liver, when we attempt to assess for marrow-derived cholangiocytes using colocalization of GFP or Y chromosome with pan-cytokeratin staining, we find that we cannot definitively identify marrow-derived epithelia cells if there are closely apposed Y chromosome–positive inflammatory cells. To address this problem, we have developed an immunofluorescence protocol to identify marrow-derived epithelial cells in the liver using co-staining for the Y chromosome and Hepatocyte Nuclear Factor-1 (HNF-1), a nuclear localized protein in liver epithelial cells (11). Using this newly developed Y FISH/HNF-1 detection method, we find rare (approximately 1 in 20,000) bone marrow–derived hepatocytes in female mice that have been transplanted with male bone marrow after lethal γ irradiation. Therefore, use of a nuclear marker of epithelial cells, such as HNF-1 in the liver or thyroid transcription factor in the lung, allows for improved discrimination of marrow-derived epithelial cells in tissue sections.

Comparison of GFP Mouse Strains

In addition, we have performed control studies in which we compared the levels of GFP expression in different cell types in the most commonly used ubiquitously expressing GFP mouse strains (10). We compared three GFP transgenic mice considered to have ubiquitously GFP expression, in which the GFP transgene is under the control of the chicken β actin (CBA) or human ubiquitin C (UBC) promoter. We characterized the expression of GFP using flow cytometry, direct tissue fluorescence, and immunostaining using commercially available anti-GFP antibodies. Mice of CBA-GFP strain "1Osb" had strong but variegated expression of GFP in adult liver, kidney, small intestine, and lung. Mice of CBA-GFP strain "Y01" had the highest proportion of GFP-positive peripheral blood cells, yet had only limited GFP expression in their solid organs. UBC-GFP mice expressed GFP very weakly in solid organs and variably in blood. We also compared different approaches for detecting GFP, and found that the best approaches are direct fluorescent detection of GFP in frozen sections, which unfortunately do not always provide adequate morphology, and immunofluorescent detection of formalin-fixed, paraffin-embedded tissue sections. Immunohistochemical staining was less sensitive than direct fluorescence or immunofluorescence, because it was subject to false positive signal in multiple tissues, most prominently in the small intestine.

Thus, we found a wide variability of GFP expression within and between GFP transgenic strains, and none of the mice tested showed truly ubiquitous GFP expression. Therefore, one must carefully analyze the pattern of GFP expression in the tissues of interest within the positive control (bone marrow donor) mouse strain when GFP is to be used as a marker of cell lineage or donor origin.

MECHANISMS FOR ENGRAFTMENT OF BONE MARROW–DERIVED EPITHELIAL CELLS

There are multiple potential mechanisms by which bone marrow–derived cells could engraft as lung epithelial cells, including (*1*) differentiation of an epithelial progenitor cell that was within the bone marrow cell population at the time of bone marrow transplantation, (*2*) transdifferentiation of a hematopoietic stem and/or progenitor cell after it is reprogrammed by the microenvironment in the injured lung, (*3*) differentiation of a pluripotent cell that was present in the bone marrow at the time of transplantation, and (*4*) fusion of a blood cell with a mature pneumocyte such that it then undergoes nuclear reprogramming. Skeletal muscle cells and osteoclasts are known to form by cell fusion, but cell fusion is otherwise considered to be very rare. However, cell fusion has been shown to be the mechanism by which bone marrow–derived cells (probably tissue macrophages) become functional hepatocytes in transgenic mice that lack the enzyme fumarylacetoacetate hydrolase (FAH), which is necessary for the normal breakdown of tyrosine (12, 13). When the therapeutic drug NTBC, which prevents the breakdown of tyrosine to its toxic metabolites, is withdrawn from FAH-null mice, the mice experience acute severe liver damage. If the mice receive wild-type (FAH+/+) bone marrow cells before withdrawal of NTBC, they can survive withdrawal of the drug because marrow-derived cells fuse with the damaged hepatocytes, and the FAH gene is expressed, which greatly ameliorates the liver disease (14).

My laboratory has examined the potential role of cell fusion in the appearance of marrow-derived epithelial cells in the lungs of mice that have undergone bone marrow transplantation after lethal irradiation. We first used a Cre-Lox system to test for fusion (15). We used donor BM from a Cre reporter strain of mice called Z/EG that switches from β-galactosidase expression to GFP expression after DNA recombination by Cre recombinase (16). The recipient mice used for these transplants

expressed Cre recombinase on the ubiquitously active chicken β-actin promoter (17). In this model, cells that formed by fusion of a donor and recipient cell would express GFP. Even cells that had fused and undergone reductive divisions (and thus were mononuclear) would be detected based on GFP expression as long as the GFP allele was maintained. In our analysis of over 25 mice, no GFP+ cells were detected in the lung, liver, GI tract, or muscle unless secondary damage was induced to the muscle or liver (which led to eGFP+ myocytes and hepatocytes, respectively). Thus, within the sensitivity of the system used, cell fusion was not required for the appearance of marrow-derived epithelial cells. Similarly, Alvarez-Dolado and colleagues (18) did not detect marrow-derived lung epithelial cells forming by cell fusion in the lung using a model in which bone marrow from a Cre reporter mouse was transplanted into lethally irradiated mice expressing Cre recombinase ubiquitously. However, in this study the dose of irradiation was lower than the threshold that induces lung injury (750 cGy), thus marrow-derived epithelial cells may not have been present.

Subsequent studies in my laboratory demonstrated that cell fusion could lead to marrow-derived epithelial cells in the lung if the lung undergoes secondary damage after bone marrow transplantation. We used targeted X-ray irradiation to reinjure the lungs of β-actin-Cre mice that had been transplanted with Z/EG BM, and then assessed for GFP expression. In this model, some bone marrow–derived type II pneumocytes were shown to have been derived by cell fusion (D.S.K. and Erica Herzog, unpublished data).

FREQUENCY OF BONE MARROW–DERIVED EPITHELIAL CELLS

As discussed above, the frequency of bone marrow–derived epithelial cells is less than 0.1% in many reported studies, and in some studies in which higher frequencies were reported, there may have been errors due to artifacts of staining that led to false positive data. That said, however, the frequency of 1 epithelial cell out of 1,000 being bone marrow derived is still much greater than zero, and could represent a large number of cells, which could have a physiologically relevant effect. It is also possible that, once we have a better understanding of the cells and mechanisms involved, we could increase the level of engraftment to assess for functional benefits. In some damage models, such as FAH-null mice, the frequency of marrow-derived cells is very much greater than 1% and can reach 50 to 80% of the hepatocytes being bone marrow derived.

Multiple variables must be considered when comparing reported frequencies of bone marrow–derived cells, including donor mice, degree of tissue damage, methods for donor cell detection, and time of analysis after tissue damage. For example, Wagers and coworkers reported that there were no bone marrow–derived lung epithelial cells and that 1 in 70,000 hepatocytes was bone marrow derived after transplantation of a single GFP+ hematopoietic stem cell from a donor mouse that expressed GFP from the constitutively active chicken β actin promoter (19). Regarding the use of the single hematopoietic stem cell that was selected based on being lineage negative, Thy1^{lo}, and expressing Sca and Kit, it is not yet clear whether this is the optimal cell to use for engrafting as epithelial cells. If all marrow-derived cells occur due to cell fusion, then this hematopoietic stem cell should be adequate in the long term for generating the fusogenic macrophages that are responsible for marrow-derived epithelial cells. However, since the donor-derived macrophages would not have been present at the same time as the acute tissue damage induced by irradiation, these fusion-derived chimeric cells may not have formed. Other publications have also reported the absence of bone marrow–derived epithelial cells in the lung, including that by Chang and colleagues (20), who showed that the cells in the lungs of their mice that had undergone bone marrow transplantation from donors with constitutive b-gal or GFP expression had what initially appeared to be bone marrow–derived type II pneumocytes, but which were actually false positive signals due to overlapping fluorescent signals of an endogenous pro–SP-C+ type II cell and a donor-derived GFP+ cell. The techniques used to assess for marrow-derived cells were rigorous—they used both costaining of tissue sections for a donor marker and SPC and also analyzed isolated lung cells on cytospins. Of note, the GFP donor mice ("1Osb") used in this study are known to have strong but variegated expression of GFP in adult liver, kidney, small intestine, and lung (10), which could affect the ability to identify rare marrow-derived epithelial cells. Another variable in this study was that the recipient mice were only 3 days old at the time of transplantation and the donor cells were administered via intraperitoneal injection. For a recent review of other publications on bone marrow to lung epithelial cells, *see* Reference 21.

CONCLUSIONS

In conclusion, studies in my laboratory and in other laboratories have confirmed that bone marrow–derived cells can take on the phenotype of epithelial cells and express epithelial-specific proteins, but these findings are dependent on multiple variables, the most important of which are the ability to identify donor-derived cells based on expression of specific genes and the degree of tissue damage induced. This work has generated much excitement and concern, and we have responded by taking into careful consideration the potential mechanisms underlying the appearance of marrow-derived epithelial cells and the development of optimal detection protocols to be able to definitively identify and quantitate marrow-derived epithelial cells. With the development of improved detection techniques, marrow-derived epithelial cells are still identified, but are more rare than we originally published, suggesting that tissue overlay had caused some false positive data. In contrast, the lack of detection of bone marrow–derived epithelial cells when using GFP as the marker of donor origin may cause an underestimation of marrow-derived epithelial cells due to variegated expression of the GFP transgene within individual epithelial cells in the organs of the transgenic mice.

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