The Role of Bone Marrow-Derived Adult Stem Cells in a Transgenic Mouse Model of Allergic Asthma

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Key Words

Asthma · Airway remodeling · Bone marrow transplantation · Mice · Fibroblasts

Abstract

Background: Asthmatic airway remodeling is an abnormal injury/repair process of the small airways caused by chronic inflammation in which the quantities of multiple cells increase dramatically. However, the origin of these proliferative cells is still undetermined. **Objective:** The aim of this study was to examine whether bone marrow (BM)-derived adult stem cells are responsible for the proliferative cells in asthmatic airway remodeling. **Methods:** Adult mice were durably engrafted with BM isolated from green fluorescent protein (GFP) transgenic mice. Using GFP BM chimera mice, an ovalbumin (OVA)-induced chronic asthma mouse model was established. The distribution of BM-derived GFP+ cells in the lungs of chronic asthma mice was detected by fluorescence microscopy. The phenotype of BM-derived GFP+ cells in the lung tissues of chronic asthma mice was analyzed by flow cytometry. **Results:** BM chimera mice were successfully generated, with no detectable radioactive inflammation observed. Using BM chimera mice, we established a mouse model of chronic asthma characterized by a significant increase in the thickness of the airway subepithelial basement

membrane and smooth muscle layers. OVA treatment caused many GFP+ cells to appear at sites of small airway inflammation. The extravascular localization of some GFP+ cells and their morphology were not consistent with leukocytes. Flow cytometric analysis of lung cells revealed a significant increase in type I collagen (Col I)+GFP+ cells and α -smooth muscle actin (α -SMA)+GFP+ cells in OVA-treated GFP BM chimera mice. **Conclusions:** Considerable numbers of Col I- and α -SMA-producing cells originated from BM in the lung tissues of mice with OVA-induced chronic asthma.

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Introduction

Asthma is a chronic airway inflammatory disease which causes airway hyperreactivity and airway remodeling. Airway remodeling in asthma has been recognized as involving structural changes in airways such as smooth muscle hypertrophy and hyperplasia, thickening and fibrosis of the subepithelial basement membrane, hypertrophy of bronchial glands, goblet cell hyperplasia and thick-

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Fig. 1. Protocol for BMT and establishment of the asthma mouse model. A total of 4×10^6 BM cells from GFP Tg or B6 mice in 200 μ l of PBS were injected into irradiated recipient B6 mice. Four weeks later, mice were sensitized with OVA/aluminum hydroxide (alum) on days 0 and 14. From day 21, mice were challenged with OVA for 20 min every day for 4 weeks.

ening of airway epithelium [1]. Airway remodeling can lead to airway hyperreactivity. Prevention and therapy of airway remodeling is important for the control of asthma.

Airway remodeling is also an abnormal injury/repair process of the small airways caused by chronic inflammation [2]. An abnormal proliferation of fibroblasts and smooth muscle cells is involved in the process [2, 3]. However, the origin of these proliferative cells is still undetermined at present. It is commonly considered that bone marrow (BM)-derived stem cells and/or endogenous tissue stem cells are responsible for the repair process of injured tissue. However, unlike some organs such as intestine and skin, the regenerative ability of lung tissue is very limited. BM-derived adult stem cells can localize to the lung and acquire phenotypic markers of lung parenchymal and interstitial cells [4, 5]. There are an increasing number of studies demonstrating a functional role of adult marrow-derived cells in lung injury. This has been described in animal models of lung inflammation, emphysema and fibrosis [6–11]. In addition, circulating fibrocytes and endothelial progenitor cells have been connected with asthmatic airway remodeling. Circulating fibrocytes may function as myofibroblast precursors and may contribute to the genesis of subepithelial fibrosis in

asthma [12]. Increased numbers of submucosal vessels in asthmatic airway remodeling are linked to the recruitment of endothelial progenitor cells [13]. So, BM-derived adult stem cells may be an important source of the proliferative cells in asthmatic airway remodeling.

In this study, we generated green fluorescent protein (GFP)+ BM chimera mice and then established an ovalbumin (OVA)-induced chronic asthma model in these mice. Flow cytometric analysis of lung cells revealed a significant increase in type I collagen (Col I)+GFP+ cells and α -smooth muscle actin (α -SMA)+GFP+ cells in OVA-treated GFP BM chimera mice compared to saline-treated controls. Thus, considerable numbers of Col I- and α -SMA-producing cells originated from BM, which shows that BM-derived adult stem cells are at least partly responsible for asthmatic airway remodeling.

Animals and Methods

Animals

Healthy female C57BL/6 mice (6–8 weeks of age) and GFP transgenic (Tg) mice on a C57BL/6 background were purchased from the experimental animal center of the Fourth Military Medical University, Xi'an, China. All mouse protocols were approved by the Animal Experiment Administration Committee of the Fourth Military Medical University.

BM Chimera Mice

GFP Tg and wild-type B6 mice were sacrificed, and BM cells were collected from femurs by aspiration and flushing. Recipient B6 mice were irradiated with ^{137}Cs (5 Gy, twice at 3-hour intervals) and were then bred under pathogen-free conditions. After irradiation, 4×10^6 BM cells from GFP Tg or B6 mice in 200 μ l of PBS were injected through the tail vein (fig. 1).

Analysis of Organ Samples from Transplant Recipients

To validate whether BM chimera mice had been successfully generated, BM, peripheral blood and spleens were collected and analyzed on days 28, 56 and 84 after BM transplantation (BMT). Briefly, peripheral blood samples were collected and then the red blood cells were lysed. Spleen samples were homogenized and depleted of red blood cells. Femoral BM cells were washed with PBS. The nucleated cells from these samples were analyzed by flow cytometry, and the number of GFP+ cells was calculated.

Mouse Model of Allergic Asthma

Four weeks after BMT, mice were sensitized with OVA according to the method of Temelkovski et al. [14] with minor modifications. A mixed suspension of 50 mg of OVA and 5 mg of aluminum hydroxide in 0.1 ml of physiologic saline was administered intraperitoneally to mice once on days 0 and 14. From day 21, mice were placed in a closed plastic inhalation chamber (length $\times$ width $\times$ height = 24 $\times$ 21 $\times$ 17 cm) and exposed to aerosol consisting of 3.0% w/v OVA suspended in physiologic saline for 20 min, every day for 4 weeks (fig. 1). Aerosol with particle diameters

Fig. 2. Creation of GFP BM chimera mice.

a Kinetics of engraftment of GFP+ BM cells. BM, peripheral blood and spleens from recipient mice were collected on days 28, 56 and 84 after transplantation with BM from GFP Tg mice. The nucleated cells from these samples were analyzed by flow cytometry, and the percentage of GFP+ cells was calculated. Data shown represent the means $\pm$ SEM from groups of 8 BM chimera mice. **b** Lung morphology of BM chimera mice. Representative lung sections from GFP BM chimera mice were stained with hematoxylin and eosin and examined by light microscopy on days 28, 56 and 84 after BMT. Scale bar = 40 μ m.

between 1 and 10 μ m was generated and administered at a flow rate of 0.3 ml/min using an ultrasonic nebulizer (402AI, Yuyue, Danyang, China) in the exposure chamber. Control animals were treated in a similar way except that the physiologic saline did not contain OVA and aluminum hydroxide.

Morphological Analysis

On day 49 after OVA sensitization, BM chimera mice were sacrificed and their lungs were obtained. For histopathology, lungs were inflated and fixed with 10% buffered formalin. Samples were embedded in paraffin and then sectioned (5 μ m). Sections were stained with hematoxylin and eosin for analysis of pathological changes. Small bronchi (integral mucosa, without cartilage) were picked out to test the thickness of the airway as previously described [15]. In brief, the airway internal perimeter, the areas of basement membrane and the areas of smooth muscle were examined with an image analyzer. The ratio of the area to the internal perimeter represented the thickness of each layer. Some sections were left unstained in order to evaluate the distribution and localization of GFP+ cells by means of fluorescence microscopy.

Analysis of Whole-Lung Cells by Flow Cytometry

On day 49 after OVA sensitization, lungs from BM chimera mice were removed and used to obtain single-cell suspensions for flow cytometric analysis as previously described [16]. Briefly, after mincing, the samples were digested with collagenase and DNase and then filtered to obtain single-cell suspensions. For staining for intracellular Col I and α -SMA, cells were permeabilized in Cytotfix/Cytoperm, washed in Perm/Wash buffer (BD Biosciences) and stained with anti-COL1A1-biotin (sc-8784, Santa Cruz Biotechnology, Santa Cruz, Calif., USA) or biotin-conjugated an-

ti- α -SMA antibody (clone 1A4, Lab Vision Corp.) for 30 min and detected by subsequent staining with streptavidin-allophycocyanin (554067, BD Biosciences). For surface staining, cells were labeled with anti-macrophage differentiation antigen-3 (Mac-3)-phycoerythrin (553324, BD Biosciences). Labeled cells were analyzed using a flow cytometer (BD FACS Calibur). The frequency of each cell subset was calculated.

Statistical Analysis

Data were expressed as means $\pm$ SEM. Statistical analyses were performed on the data using single-factor ANOVA for the three groups and Student's unpaired t test for comparisons of two groups. $p < 0.05$ was considered statistically significant.

Results

Creation of GFP BM Chimera Mice

To confirm that BM chimera mice had been successfully generated, we examined the relative ratio of GFP+ cells by flow cytometry in BM, peripheral blood and spleen samples on days 28, 56 and 84 after BMT. On day 28, more than 93% of BM and blood cells were GFP+, while only more than 70% were GFP+ in spleen. On days 56 and 84, the percentages of GFP+ cells in both BM and peripheral blood remained unchanged (fig. 2a; BM: $p > 0.05$ on days 56 and 84; blood: $p > 0.05$ on days 56 and 84), while the level in spleen increased to more than 86

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Fig. 3. Phenotype analysis of the chronic asthma model in GFP BM chimera mice. **a** Lung morphology of BM chimera mice. Representative lung sections were stained with hematoxylin and eosin and examined by light microscopy. Scale bar = 40 μm . **b** Thickness of small airway basement membrane and smooth muscle. Data shown represent the means $\pm$ SEM from groups of 10 BM chimera mice. PS = Physiologic saline.

and 87%, respectively (fig. 2a; $p < 0.05$ on days 56 and 84). These data indicated that the BM of recipient mice had been adequately destroyed by irradiation and that BM reconstitution was successful.

Because irradiation may induce radiation pneumonia that may influence the end points to be observed in the asthmatic airway remodeling model, lung histological sections were compared from GFP BM chimera mice on days 28, 56 and 84 after BMT. Hematoxylin and eosin staining was used to detect lung histological architecture. Our results showed no evidence of inflammation or fibrosis on days 28, 56 and 84 after BMT (fig. 2b). Thus, our irradiation protocol had no significant effects on the lung morphology of BM chimera mice.

Establishment of the Chronic Asthma Model

Using BM chimera mice, we established a mouse model of asthma through repetitive OVA exposure over a 4-week period. We evaluated the lung histology of chimera mice after chronic OVA exposure. As shown in figure 3a, repetitive OVA exposure resulted in significant increases in the amount of peribronchial and perivascular infiltration by inflammatory cells into the airways of OVA-treated mice as compared with saline-treated mice. Moreover, OVA exposure induced significant increases

in the thickness of the subepithelial basement membrane and smooth muscle layers in the airways of OVA-treated mice compared with saline-treated mice (fig. 3b; $p < 0.05$ for subepithelial basement membrane and smooth muscle layer). These histological changes in lungs from OVA-treated GFP BM chimera mice were comparable to those in OVA-treated B6 mice transplanted with wild-type B6 BM (data not shown). Therefore, a chronic asthma model characterized by airway remodeling was established in BM chimera mice.

Distribution of BM-Derived GFP+ Cells in the Lung Tissues of Asthmatic Mice

To investigate whether BM-derived GFP+ cells were involved in the pathogenesis of asthma, we observed the distribution of GFP+ cells in the lung tissues of both OVA- and saline-treated GFP BM chimera mice by fluorescence microscopy. Our results showed that in OVA-treated mice, many cells expressed GFP, and they were especially densely clustered at the sites of small airway inflammation (fig. 4). In contrast, lungs from saline-treated mice showed very few GFP+ cells (fig. 4). Thus, BM-derived GFP+ cells may participate in the pathogenesis of asthma.

Fig. 4. BM-derived GFP+ cells in the lung tissues of chronic asthma mice. Representative lung sections were examined by fluorescence microscopy. PS = Physiologic saline. Scale bar = 40 μ m.

Phenotype Analysis of BM-Derived GFP+ Cells in the Lung Tissues of Asthmatic Mice

Asthma is an immunological disease, and many infiltrating GFP+ cells observed in OVA-treated BM chimera mice should be represented by immune/inflammatory cells. To investigate whether additional lung parenchymal cell types may also be derived from the BM, whole-lung cells were isolated from OVA- and saline-treated GFP BM chimera mice on day 49 after OVA sensitization and then analyzed by flow cytometry. Because the deposition of Col I and increased smooth muscle mass are key characteristic findings in the lung tissue of mice with chronic asthma [1], the populations of cells producing Col I and α -SMA in whole-lung cells were examined. Our results confirmed that more than 23.2% of the Col I+ cells and 7.3% of the α -SMA+ cells were GFP+ in OVA-treated GFP BM chimera mice (fig. 5a). However, only 10.0% of the Col I+ cells and 2.0% of the α -SMA+ cells were GFP+ in saline-treated controls (fig. 5a; $p < 0.05$ for Col I and α -SMA). These data demonstrated that BM-derived cells represent a proportion of the proliferative Col I- and α -SMA-producing cells in lung tissues of mice with OVA-induced chronic asthma and thus probably play an important role in asthmatic airway remodeling. To rule out the contribution of BM-derived macrophages, which could

Fig. 5. Phenotype analysis of BM-derived GFP+ cells in the lung tissues of chronic asthma mice. **a** Expression of Col I and α -SMA in whole-lung cells. Representative flow cytometric analysis and percentages of cells in indicated squares are shown. Data shown represent the means $\pm$ SEM from groups of 8 BM chimera mice. **b** Expression of MAC-3 in whole-lung cells. Representative flow cytometric analysis and percentages of cells in indicated squares are shown. PS = Physiologic saline.

have taken up degraded collagen and α -SMA, the population of cells expressing MAC-3, which is a specific marker for macrophages, was also evaluated in whole-lung cells. Only $7.9 \pm 0.8\%$ of Col I+ cells and $2.9 \pm 0.4\%$ of α -SMA+ cells expressed MAC-3 (fig. 5b). Thus, at least 15.3% of Col I-producing cells and 4.4% of α -SMA-producing cells in the lung tissues of mice with OVA-induced chronic asthma originated from BM.

Discussion

In this study, we demonstrated that considerable numbers of Col I- and α -SMA-producing cells in the lung tissues of mice with OVA-induced chronic asthma originated from BM. Thus, BM-derived adult stem cells are at least partly responsible for the proliferative cells in asthmatic airway remodeling. In the past several decades, many studies have suggested that multiple immune cells, lung parenchyma cells, many kinds of cytokines and inflammatory mediators interact with each other and form a complex network which contributes to the development of airway remodeling. From a special viewpoint – the origin of proliferative cells – our findings have enriched our understanding of the mechanisms of asthmatic airway remodeling.

Airway remodeling contributes to the worsening of airway narrowing, airflow obstruction and disease progression [16, 17]. Airway wall thickness has been associated with the severity of asthma, and in severe asthma, increased airway thickness is observed [18]. Thus, airway remodeling is associated with a poorer clinical outcome among patients with asthma. However, at present, there are no medical measures which can effectively inhibit airway remodeling. Investigating the possible role of BM-derived adult stem cells in asthmatic airway remodeling may provide some new clues for the treatment of asthmatic airway remodeling. In this study, we used a GFP+ BM chimera mouse model to examine whether BM-derived adult stem cells are involved in asthmatic airway remodeling.

Adult mice were irradiated to deplete BM cells and then transplanted with BM cells bearing the GFP marker, which permits donor cell identification after transfer into receptor mice. However, because irradiation may induce radiation pneumonia, we carefully decreased the radiation dose and divided the total dose into two doses as previously described by Hashimoto et al. [19]. Our results showed that no detectable inflammation was observed, and the BM chimera mice achieved normal body weight by day 84 after BMT.

Then, using GFP BM chimera mice, we established a mouse model of allergic asthma through repetitive OVA exposure over a 4-week period. Lung histology studies revealed peribronchial and perivascular infiltration by inflammatory cells, which indicated that airway inflammation was successfully induced by OVA exposure. The ongoing inflammation can contribute to airway structural changes such as subepithelial fibrosis and increased smooth muscle mass, namely airway remodeling. Indeed, we observed that repetitive OVA exposure induced significant increases in the thickness of the subepithelial basement membrane and smooth muscle layers in the airways of OVA-treated mice.

Next, we examined the distribution of GFP+ cells in lung tissue sections by fluorescence microscopy. The number of GFP+ cells in lung tissue was vastly increased in OVA-treated chimera mice compared to saline-treated chimera mice. The intravascular GFP+ cells would be leukocytes and the epithelial GFP+ cells would be alveolar cells. It has been reported that BM-derived adult stem cells could be recruited to lung tissue and acquire the phenotype of alveolar epithelial cells [20]. However, in our OVA-treated chimera mice, the extravascular localization of some GFP+ cells, as well as their morphology, was not consistent with leukocytes or alveolar cells. Flow cytometric analysis confirmed that more than 23.2% of the Col I+ cells and 7.3% of the α -SMA+ cells were GFP+ in OVA-treated GFP BM chimera mice. In contrast, only 10.0% of the Col I+ cells and 2.0% of the α -SMA+ cells were GFP+ in saline-treated controls. Thus, BM-derived cells represent a proportion of the proliferative Col I- and α -SMA-producing cells in lung tissues of mice with OVA-induced chronic asthma.

Col I has been identified as a marker of fibroblasts. Fibroblasts represent the key source of interstitial collagens, including collagens I, III and V, fibronectin and tenascin. Increased deposition of these collagen proteins can lead to thickening of the basement membrane underneath the epithelium, which is an important feature of asthmatic airway remodeling [21]. Fibroblasts are known to be heterogeneous with respect to a number of phenotypic features, and the BM origin of fibroblasts has been confirmed [19]. Here, we provide direct evidence that BM-derived adult stem cells can relocate to the lung tissues of chronic asthma mice and present a phenotype expressing Col I. However, the phenotype and function of the Col I+GFP+ cells are still unclear, and we are actively investigating this at present.

We also found that the proportion of α -SMA+GFP+ cells in α -SMA+ cells was significantly increased in OVA-

treated GFP BM chimera mice (fig. 5a). Airway smooth muscle cells, myofibroblasts and vascular smooth muscle cells express α -SMA. In asthmatic mice, the airway smooth muscle mass increased markedly due to a coordinated increase in the size (hypertrophy) and number (hyperplasia) of airway smooth muscle cells. Airway smooth muscle cells are known to actively participate in the inflammatory and remodeling processes through their release of proinflammatory cytokines, chemokines and extracellular matrix proteins and may therefore contribute to the pathogenesis of asthmatic airway remodeling [22–24]. However, at present very little is known regarding the origin and turnover of airway smooth muscle [25]. Our data indicated that BM-derived adult stem cells may be a progenitor of proliferative airway smooth muscle cells in asthma.

In summary, we have shown that considerable numbers of proliferative Col I- and α -SMA-producing cells in

the development of asthmatic airway remodeling originated from BM. This finding directly demonstrates the importance of BM-derived adult stem cells in the pathogenesis of asthmatic airway remodeling in a mouse model. However, the exact migratory and differentiated mechanisms of these BM-derived Col I- and α -SMA-producing cells still await investigation. In addition, whether a similar phenomenon could be observed in patients with asthma who had accepted BMT is also a very interesting problem. Together with our results, further relevant studies may explore some effective measures to control asthmatic airway remodeling.

Financial Disclosure and Conflicts of Interest

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