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Anti-fibrotic Effects of Reserpine on Lung Fibrosis: Stem Cells in the Pathogenesis of Pneumofibrosis

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Abstract: In this study was found that inflammation in interstitial lung tissue of mice C57Bl/6 after intratracheal injection of the bleomycin is accompanied by the increase of the bone marrow and circulating blood cells with the phenotype (CD3, CD45R (B220), Ly6C, Ly6G (Gr1), CD11b (Mac1), TER-119), Sca-1^+, c-Kit^+, $CD34^-$ (hematopoietic stem cells (HSCs)) and hematopoietic progenitor cells (granulocyte-erythroid-macrophage-megakaryocytic (CFU-GEMM) and granulocyte precursors (CFU-G)). These results demonstrate that the intraperitoneal reserpine injection reduces alveolar interstitium and alveolar passages infiltration by inflammatory cells and prevents the connective tissue growth in the lung parenchyma. Supposedly, the reserpine anti-inflammatory effect is caused by the reduced activity of the bone marrow HSCs differentiation in CFU-G, the decrease of circulating HSCs and progenitor hematopoietic cells and the violation of their migration in bleomycin-treated lungs. These data suggest the decrease caused by the reserpine in deposition of collagen fibers in the lung's parenchyma associated with the decrease in the inflow of multipotent mesenchymal stromal cells (MSCs) and fibroblast progenitor bone marrow-derived cells to lungs. Thus, reserpine violates the MSCs differentiation into fibroblast-like cells.

Keywords: Pulmonary fibrosis, reserpine, hematopoietic stem cell, multipotent mesenchymal stromal cells.

BACKGROUND

Idiopathic pulmonary fibrosis (IPF) is a chronic active progressive disease of the unknown etiology. IPF prognosis is mostly unfavorable, the patient's health status gets worse rapidly. Life expectancy after diagnosis is 2 or 4 years. The existing set of medical actions for pulmonary fibrosis is limited and ineffective. Clinical practice is focused primarily on the treatment of complications and the maintenance therapy [1].

Recently, the attention is focused on the use of stem cells for tissue regeneration [2, 3]. For example, cell therapy using multipotent mesenchymal stromal cells (MSCs) is regarded as a perspective method for the treatment of chronic lung diseases such as pulmonary fibrosis [3, 4]. In particular, the injection of donor MSCs reduces the bleomycin-induced lung injury in mice and the deposition of collagen leads to increase the number of alveolar-epithelial cells [5-7]. The results of some experimental studies with a certain probability permit to suggest the bone marrow origin of lung cells, that express the pan-hematopoietic marker CD45 [8]. Hematopoietic cells that may have recruited to the lungs from the bone marrow supported bleomycin-initiated collagen production by releasing profibrotic cytokines [9]. Remarkable that the authors use the graft material in most of experiments performed to prove the involvement of bone marrow stem cells in fibrotic changes in the lung. In our opinion, this approach does not allow to form a reliable picture about the role of endogenous stem cells in the pathogenesis of idiopathic pulmonary fibrosis.

In previous experimental researches, we have demonstrated the principal possibility of reserpine treatment of cyclophosphamide-induced myelosuppression [10]. The effect of reserpine was largely related to its influence on hematopoietic stem cells (HSCs) and hematopoietic progenitor cells [11, 12]. Some researchers connect the synthesis of collagen fibers in the lungs with the activity of adrenergic system [13, 14]. Based on this, we have suggested, that compounds, that reduce the activity of the adrenergic system, may have anti-fibrotic effect in fibrotic lung.

In view of this, we have attempted to examine the role of endogenous HSCs and hematopoietic progenitor cells in the pathogenesis of IPF in mice C57BL/6 after intratracheal (IT) administration of bleomycin. We investigated the effects of intraperitoneal (ip) injection of reserpine on the development of pulmonary fibrosis and the possible link of reserpine's effects with the endogenous MSCs, HSCs and progenitor cells.

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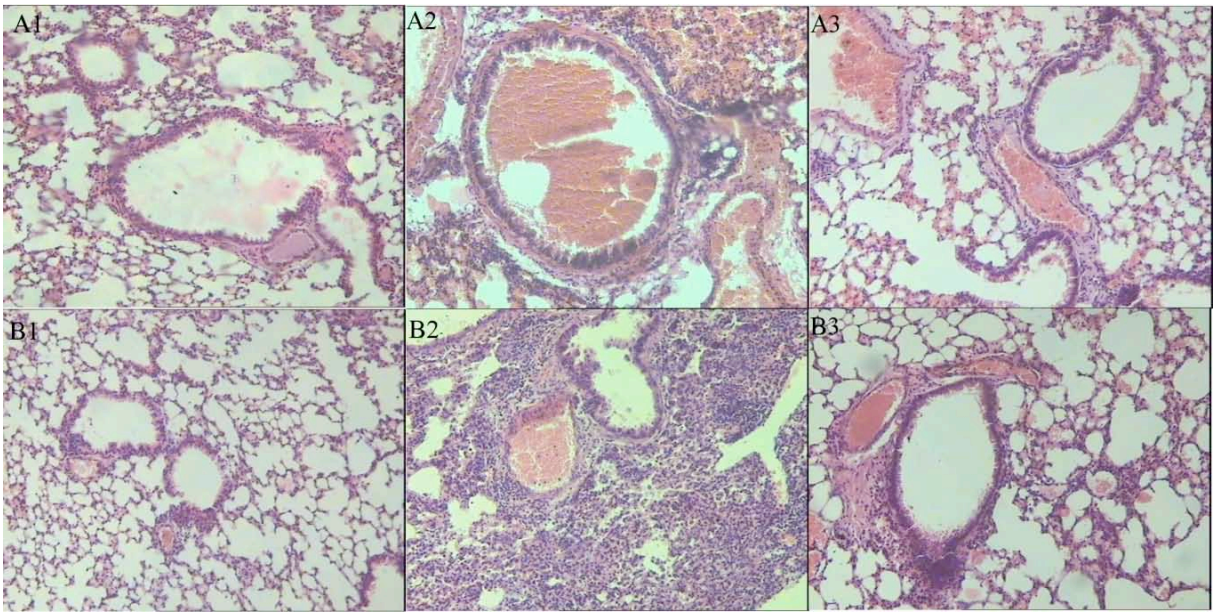

Fig. (1). Photomicrographs of representative lung sections obtained from mice C57Bl/6 on the 7th (**A**) and 21st (**B**) days of the experiment. Tissues were stained with haematoxylin and eosin in order to investigate inflammatory cells accumulation. (**A1, B1)** Mice receiving IT NaCl, (**A2**, **B2)** Mice receiving IT bleomycin, **(A3, B3)** Mice with fibrosis, treated with reserpine. Original magnifications = 300x.

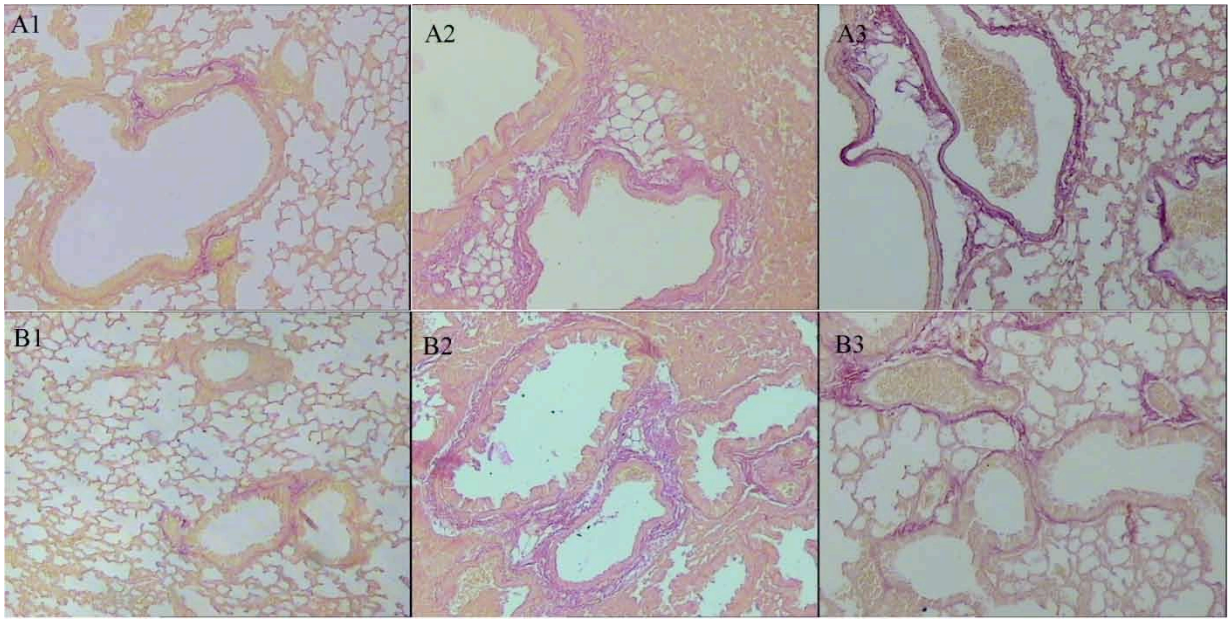

Fig. (2). Photomicrographs of representative lung sections obtained from mice C57Bl/6 on the 7th **(A**) and 21st (**B**) days of the experiment. Tissues were stained with picrofuchsin to determine the collagen content. (**A1, B1)** Mice receiving IT NaCl, (**A2**, **B2)** Mice receiving IT bleomycin, **(A3, B3)** Mice with fibrosis, treated with reserpine. Original magnifications = 100x.

These studies for the first time demonstrated that the inflammation, induced by bleomycin in the lung develops on the background of clonal activity increase of bone marrow, circulating stem cells and hematopoietic progenitor cells. Some bone marrow HSCs selectively differentiate into granulocyte progenitor cells. Reserpine therapy reduces the bleomycin-induced fibrosis. The main anti-fibrotic reserpine effects are the decrease in the activity of differentiation of bone marrow HSCs in the progenitor granulocyte cells, the reduction of bone marrow and circulating progenitor hematopoietic cells, and the possible violation of their migration to bleo-treated lungs. Besides, reserpine contributes to lower lung MSCs differentiation in fibroblast-like cells, blocking the inflow of bone marrow progenitor fibroblast cells in the interstitial tissue of the lungs.

RESULTS

Histopathological Assay of Lungs

Figs. (**1** and **2**) illustrate the morphology of the mice lungs after intratracheal (IT) NaCl inoculation, mice after IT inoculation of bleomycin and reserpine treatment and mice after IT inoculation of bleomycin and NaCl treatment. Mice were treated with bleomycin on day 0. The lungs were extracted and histopathologically investigated on 3, 7, 14, 21, 28, 40, 60 days after bleomycin. As expected, bleomycin

Table 1. The Content of the Connective Tissue in the Lungs of Mice C57BL / 6 (% of the Area of Lung Tissue) (M ± m)

Periods of the Investigation, Day	Mice Receiving IT NaCl	Mice with Fibrosis, Treated with NaCl	Mice with Fibrosis, Treated with Reserpine
7	1.13 ± 0.07	1.98 ± 0.12 *	1.33 ± 0.06 * &
14	1.14 ± 0.05	3.09 ± 0.23 *	2.94 ± 0.27 *
21	1.03 ± 0.06	5.45 ± 0.74 *	3.46 ± 0.58 * &
28	1.14 ± 0.03	3.02 ± 0.44 *	2.75 ± 0.42 *
40	1.12 ± 0.02	3.47 ± 0.21 *	2.58 ± 0.30 *
60	1.03 ± 0.09	2.77 ± 0.25 *	1.59 ± 0.39 &

Note. * - Significance of the difference with the mice, that received IT NaCl ($P < 0.05$), & - significance of the difference with the mice, that received IT bleomycin and treated with NaCl ($P<0.05$).

on the 3rd day caused venous hyperemia in the walls of the alveoli and edema of interalveolar septa epithelium compared to the NaCl-treated mice. Staining of samples with haematoxylin and eosin revealed the peribronchial inflammatory infiltrates around large vessels, contained lymphocytes, histiocytes, alveolar macrophages, neutrophils and eosinophils in the bleo-lungs. On the 7th day hyperemia and edema amplified, exfoliated alveolocytes appeared in the lumina of the alveoli (Fig. **1**, **2**).

During this period, the intensity of the increased interstitial inflammation, infiltration of inflammatory cells leads to a thickening of the alveolar walls. Van Gieson stain method with pikrofuksin revealed deposition of collagen fibers in the lung tissue of mice, around the vessels and peribronchial. On the 14th day after the bleomycin injection edema of tissue increased, sero-fibrinous exudate appeared in the alveoli, the number of desquamated alveolocytes and infiltrating cells increased. There were areas without pulmonary pattern at lung root. The small cysts were formed in the bronchioles and alveolar structure was destroyed. On the 21st day the number of mononuclear cells, attracted to the lungs with bleomycin, reached the maximum, "honeycombing lung" was formed (Fig. **1**, **2**). The collagen fibers deposition lasted on the 28th, 40th, 60th days of the experiment (Table **1**). The most expressed fibrogenic response to bleomycin injection was observed on the 28th day.

Treatment with reserpine led to decrease the edema of epithelium and hyperemia of the alveolar walls in bleo-lungs throughout the study period (compared to mice treated with bleomycin and NaCl). In addition, the airiness of lung tissue persisted and cellular infiltration was predominantly peribronchial and perivascular. In contrast to mice treated with bleomycin and NaCl, there was a minor yield of inflammatory cells into the lumen of the alveoli in a group with reserpine (Fig. **1**, **2**).

Thus, sympatholytic reserpine leads to impaired development of alveolitis and fibrosis of interstitial lung tissue of mice in conditions of the intratracheal administration of bleomycin.

Morphological Assay of the Blood and Bone Marrow

The morphological investigation revealed increase of the absolute number of neutrophils in the blood of mice with fibrosis treated with NaCl on 3, 7, 14, 21, 28, 40, 60 days compared histologically healthy animals (IT injection of NaCl) (Fig. **3**). On the 21st and 40th day, improvement rate was the higest. The increase of absolute neutrophil counts in mice treated with bleomycin and NaCl was observed in the bone marrow on the 3rd, 7th and 28th days. In addition, the content of lymphocytes increased in the bone marrow (7th, 14th, 60th days).

Reserpine reduced the absolute number of neutrophils in the bone marrow (3rd, 7th, 28th days) and peripheral blood (3rd, 7th, 21st, 40th days) in bleo-treated mice compared to mice treated with bleomycin and NaCl (Fig. **3**). Reserpine exerted suppressive effect on lymphocytes of bone marrow (7th, 14th, 60th days) and blood (3rd, 7th, 14th, 60th days).

These data showed that the increase in the absolute number of neutrophils and lymphocytes in the bone marrow and leukocytosis in the peripheral blood was observed in mice of the C57Bl / 6 on the background of diffuse pneumofibrosis. Number of neutrophils and lymphocytes decreases in the bone marrow and blood of fibrotic animals under the reserpine action.

Immunophenotypic Characterization of Mononuclear Cells, Presented in Bone Marrow and Lungs

Hematopoietic Stem Cells

On the 7th day of the experiment we studied the phenotype of nonadherent cells by the protocol for hematopoietic stem cells (BD Biosciences, US). Cells for this experiment were derived from the bone marrow and lungs of histologically healthy animals, of mice with IT administration of bleomycin and treated NaCl, and of fibrotic mice, treated with reserpine. Surface phenotype of mononuclear cells, obtained from bone marrow and lungs of all mice, was negative for markers of mature hematopoietic cell populations (CD3, CD45R (B220), Ly6C and Ly6G (Gr1), CD11b, TER-119) and was positive for Sca-1, c-Kit and negative for CD34 (Fig. **4**). Quantitative analysis of cells on the 7th experimental day showed that absolute number of HSCs in bleomycin-injured lungs of animals and treated with NaCl (0.013%), decreased 2-fold compared with histologically healthy animals (0.02%). Reserpine, however, increased the number of HSCs in the bleo-treated lungs to the level of healthy animals (0.02%) (Fig: **4**).

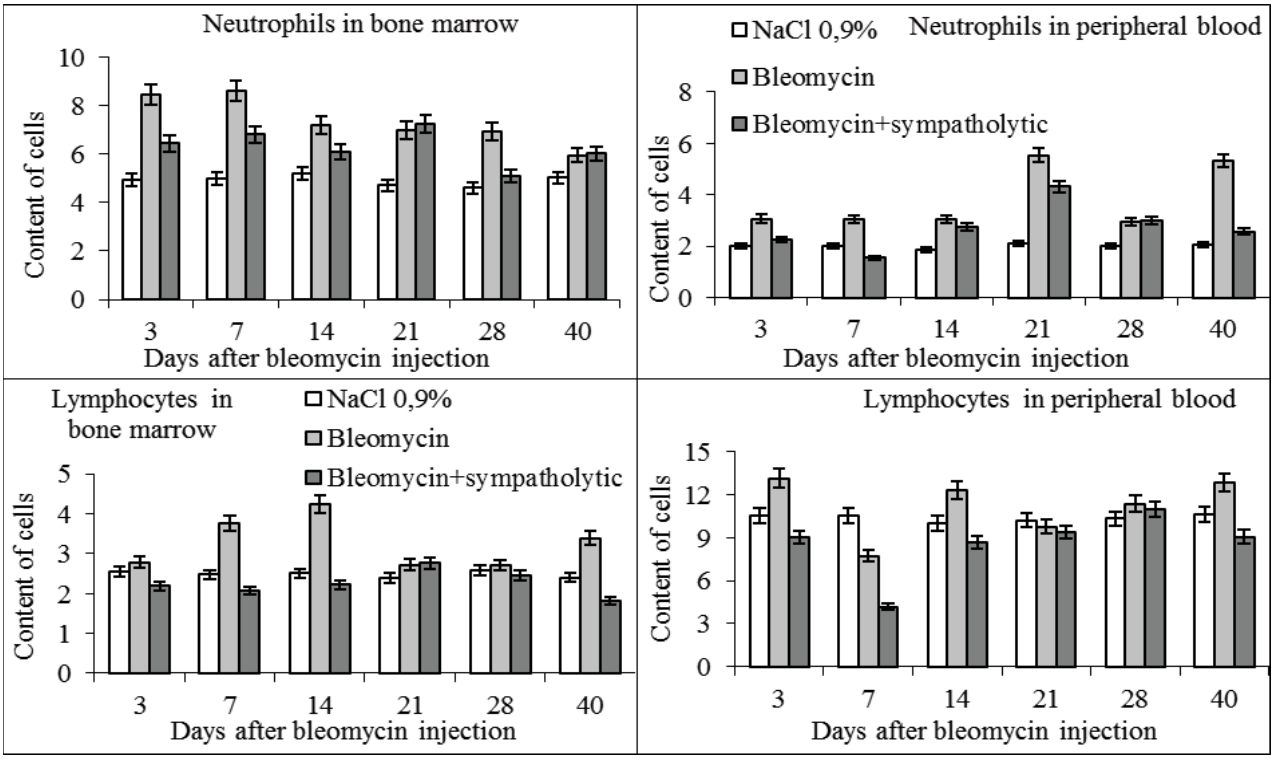

Fig. (3). The content of neutrophils and lymphocytes in the bone marrow and in the peripheral blood of mice C57BL/6 after bleomycin injection: Mice received IT of NaCl 0.9% (**NaCl 0.9%**), mice received IT of bleomycin (**Bleomycin**), mice received reserpine after intratracheal administration of bleomycin (**Bleomycin+sympatholytic**). Values represent means ± SEM, parametric t test and nonparametric Mann-Whitney test were used.

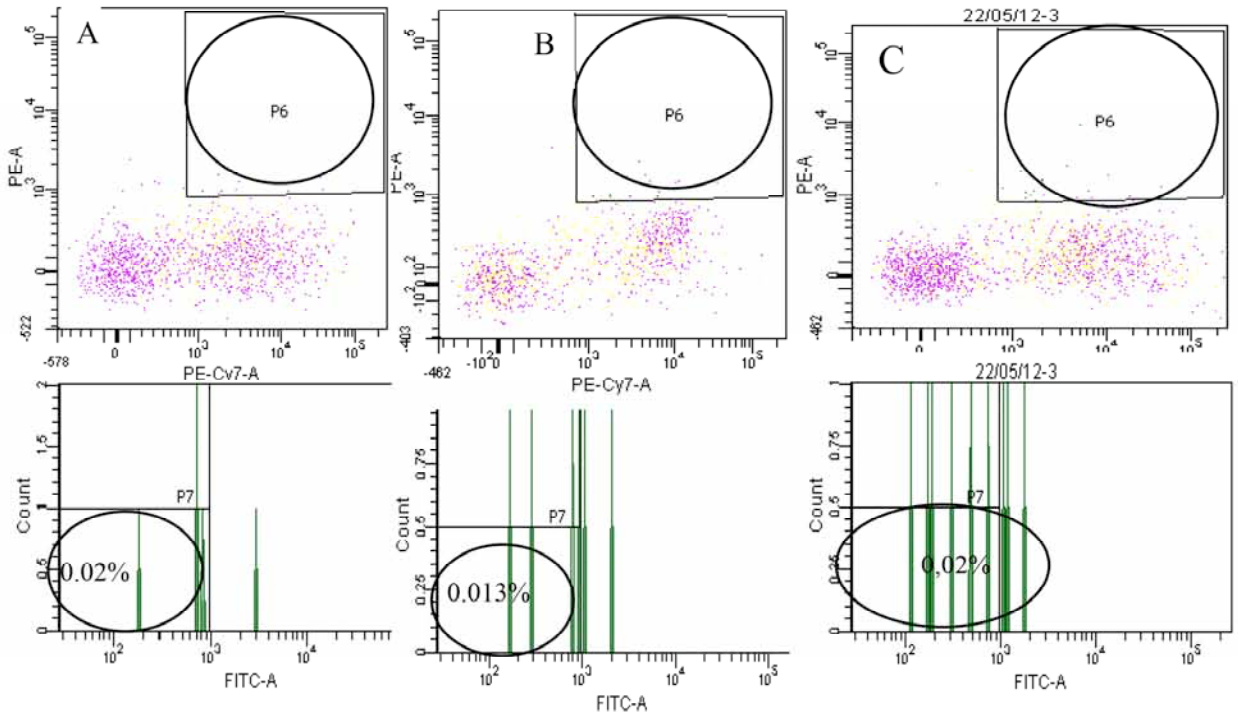

Fig. (4). Flow cytometric assay of cell-surface antigens on hematopoietic stem cells from lungs of mice with intratracheal administration of NaCl 0.9% (**A**), mice with intratracheal administration of bleomycin (**B**), mice with intratracheal administration of bleomycin and treated with reserpine (**C**) (7^{th} day of the experiment). The phenotype of cells from lung was studied on the protocol for hematopoietic stem cells (BD Biosciences, US). The phenotype of cells was Lin-, Sca-1+, c-Kit+, CD34-. A, B and C percentages represent the mean from 3 different samples.

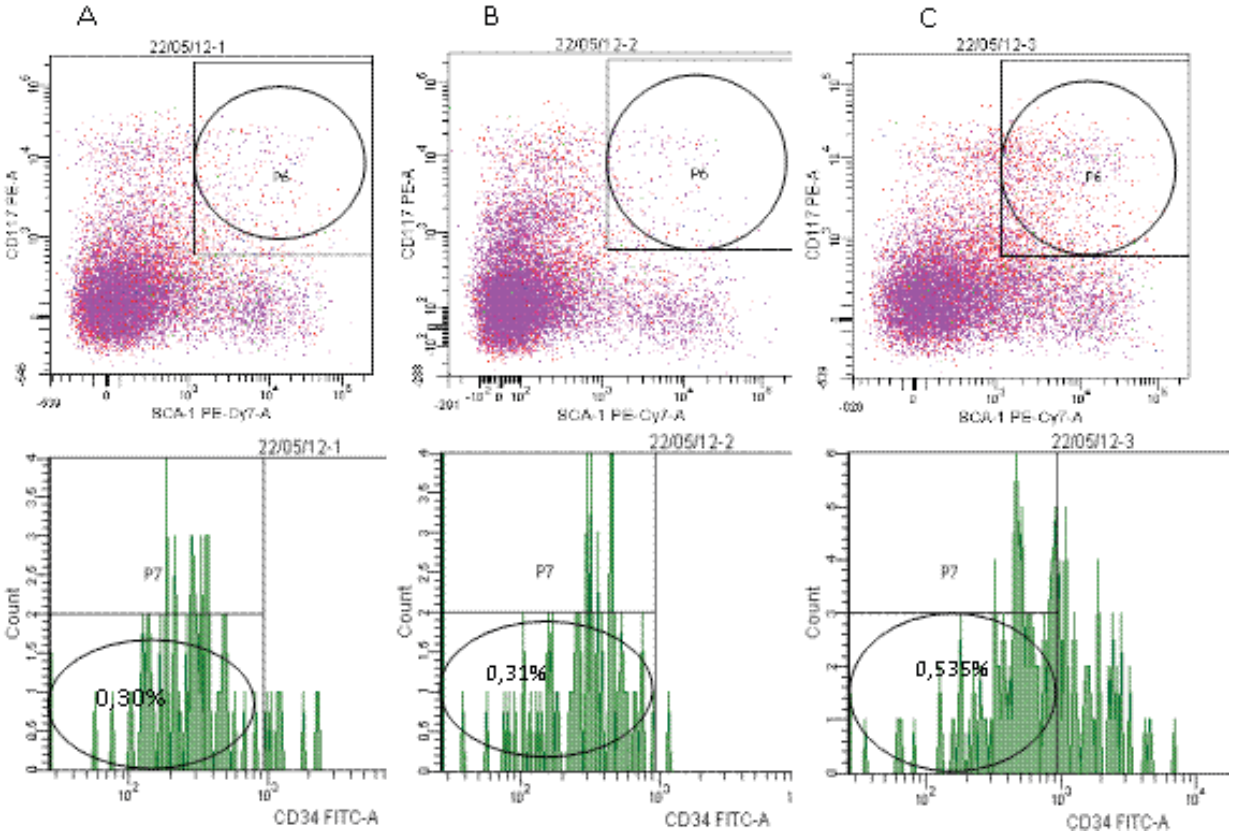

Fig. (5). Flow cytometric assay of cell-surface antigens on hematopoietic stem cells from bone marrow of mice with intratracheal administration of NaCl 0.9% (**A**), mice with intratracheal administration of bleomycin (**B**), mice with intratracheal administration of bleomycin and treated with reserpine (**C**) (7th day of the experiment). The phenotype of cells from bone marrow was studied on the protocol for hematopoietic stem cells (BD Biosciences, US). The phenotype of cells was Lin-, Sca-1+, c-Kit+, CD34-. A, B and C percentages represent the mean from 3 different samples.

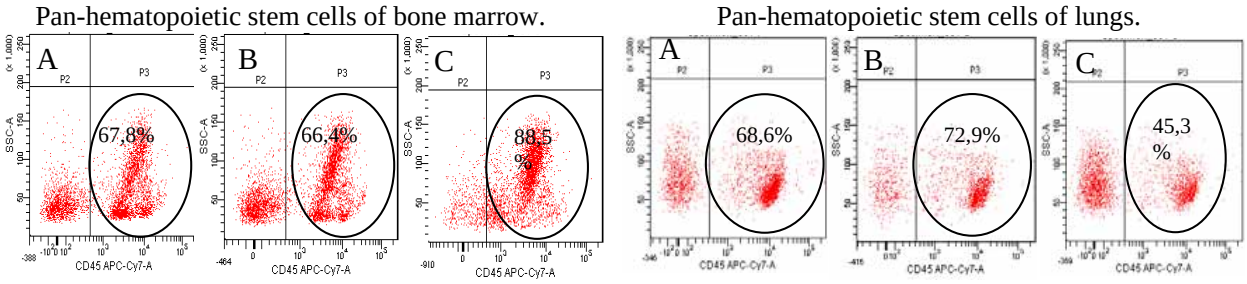

Fig. (6). Flow cytometric assay of cell-surface antigens on murine pan-hematopoietic stem cells. The figure plots show the number of CD45+ cells (percentage of labeled cells). (A) mice with intratracheal administration of NaCl 0.9%, (B) mice with intratracheal administration of bleomycin, (C) mice with intratracheal administration of bleomycin and treated with reserpine (21st day of the experiment). A, B and C percentages represent the mean from 3 different samples.

The amount of HSCs was 0.31% of the total number of studied nonadherent mononuclear cells in bone marrow of fibrosis mice, treated with NaCl.

The amount of HSCs in the bone marrow of mice with IT injection of NaCl was 0.30% of the total number of studied nonadherent mononuclear cells. The index increased 1.73 times in group of animals treated with reserpine, compared to mice with IT bleomycin or NaCl (Fig. **5**).

Thus, we suggest that bleomycin reduced the HSC amount in the bone marrow and lungs of mice on the 7th day of the experiment. Reserpine, however, caused its increase in the studied tissues.

Pan-hematopoietic Cells

On the 21st day of the experiment $CD45^+$ cells (pan-hematopoietic cells) from the bone marrow and lungs were measured by flow cytometry. The results of FACS of mice are shown in Fig. (**6**). In histologically healthy mice 67.8% of all labeled nonadherent bone marrow cells were $CD45^+$. In fibrotic mice this cell population was 66.4%. Fraction of pan-haematopoietic $CD45^+$ cells in the bone marrow of fibrotic mice, treated with reserpine, was increased to 88.5%.

Number of CD45 pos cells in the fraction of nonadherent lung cells of mice that received IT NaCl (68.6%), and of mice with fibrosis (72.9%) did not differ significantly from

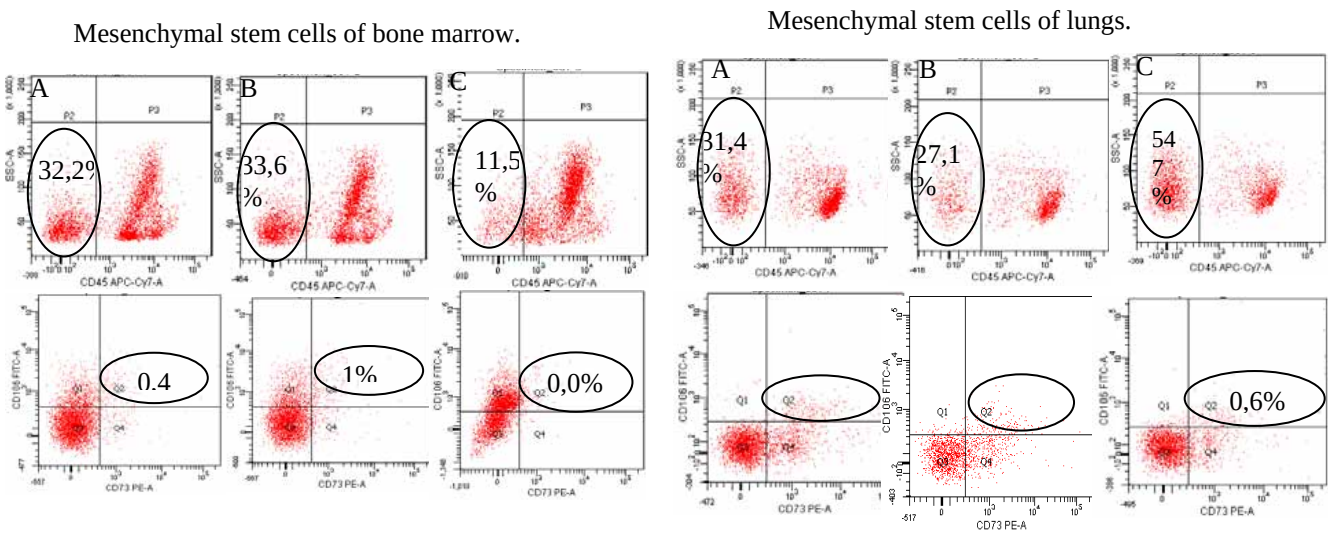

Fig. (7). Flow cytometric analysis of cell-surface antigens on murine mesenchymal stem cells from bone marrow and lungs, derived on the 21st day of experiment. The dot plots for cell suspensions labeled with antibodies directed against hematopoietic (CD45) cells in the adherent cell fraction. CD73+ and CD106+ are detected in the CD45- fraction. (**A**) Mice with intratracheal administration of NaCl 0.9%, (**B**) mice with intratracheal administration of bleomycin, (**C**) mice with intratracheal administration of bleomycin, treated with reserpine. A, B and C percentages represent the mean from 3 different samples.

each other (Fig. **6**). Population of haematopoietic CD45 + cells in the lungs of fibrotic mice treated with reserpine was 45.3% of all labeled nonadherent cells.

These data showed that bleomycin almost had no influence on the content of CD45 pos cells in the bone marrow and lungs of mice on the 21st day experience. Reserpine increased the number of pan-hematopoietic cells in the bone marrow and reduced in fibrotic lung parenchyma.

Mesenchymal Multipotent Stem Cells

On the 21st day of the experiment the population of CD45 neg cells was determined in the bone marrow and lungs, the expression of CD31 (Platelet Endothelial Cell Adhesion Molecule-1), CD34 (stem / progenitor hematopoietic cells), CD44 (Hyaluronate receptor), CD73 (Ecto -5'-nucleotidase), CD90 (Thy-1 glycoprotein) and CD106 (Vascular cell adhesion molecule-1) on their surface was evaluated. It turned out that the population of CD45 neg cells was 32.2% of all the labeled adherent cells from bone marrow of histologically healthy mice, from fibrotic mice with NaCl-33.6% (Fig. **7**) and decreased to 11.5% in the bone marrow of mice with pneumofibrosis treated with reserpine. According to data of Chow K.S. *et al*. the share of CD45neg cells in the lungs of healthy mice is in the range of 30-40% of all labeled mononuclear [15]. In our studies, the proportion of CD45-cells in the healthy lungs makes to 31.4% and in fibrotic lungs of animals with NaCl - 27.1%. At the same time, in fibrotic lungs of mice treated with reserpine, the proportion of CD45-cells from all the labeled cells increased to 54.7%.

CD45- cells of lungs and bone marrow from mice of all groups were expressed on the surface of CD44, CD73, CD90 and CD106. At the same time CD45-cells were negative by CD31 and CD34. The revealed immunophenotypic characterization of mononuclear was inherent to MSCs of mice lungs [16]. Quantitative analysis showed that at group of histologically normal mice 1.7% of all allocated CD45 neg lung cells were defined as subpopulation with $CD45^-$, $CD73^+$, $CD106^+$ (Fig. **7**). In the fraction of CD45-cell of fibrotic lung in mice with NaCl this population was 2%. In fibrotic mice, treated with reserpine, the number of these cells was 0.6% of the population of CD45 neg cells.

On the 21st day of the experiment subpopulation of cells (CD44, CD73, CD90, CD106 positive and CD31, CD34, CD45 negative) was found in the bone marrow of healthy mice (0.4% of all CD45 neg cells) and in the bone marrow of mice with fibrosis and treated with NaCl (1% of all CD45 neg cells) (Fig. **7**). Noteworthy the cells with this phenotype are not detected in the bone marrow of fibrotic mice, treated with reserpine.

These observations have shown that bleomycin has no significant effect on the CD45 neg cells of bone marrow and lungs of mice C57BL / 6 on the 21st day of the experiment, against this background, amount of MSCs increased in the examined tissue. Reserpine had a suppressive effect on the bone marrow CD45- cells, including the subpopulation of cells with the phenotype CD44+, CD73+, CD90+, CD106+, CD31-, CD34-, CD45-. At the same time, the population of the fibrotic lung CD45 neg cells was expanded, and the number of MSCs, on the contrary, was decreased under the influence of reserpine.

Multilineage Differentiation of Pulmonary MSCs in vitro on the 21st day of Pneumofibrosis

Adherent cells were obtained after secondary culturing of control groups (histologically healthy mice, the mice with fibrosis and treated with NaCl) and experimental group (mice with fibrosis, treated with reserpine). They were maintained with a stable morphology of fibroblasts, expressing on the surface CD44, CD73, CD90, CD106, but do not expressing CD31, CD34, CD45 (MSC-like cells). Osteogenesis, adipogenesis, chondrogenesis and differentiation of mononuclear cells with mesenchymal phenotype, were perfomed by using standard protocols [17]. MSC-like cells of histologically healthy mice and of mice with fibrosis under the influence of appropriate growth

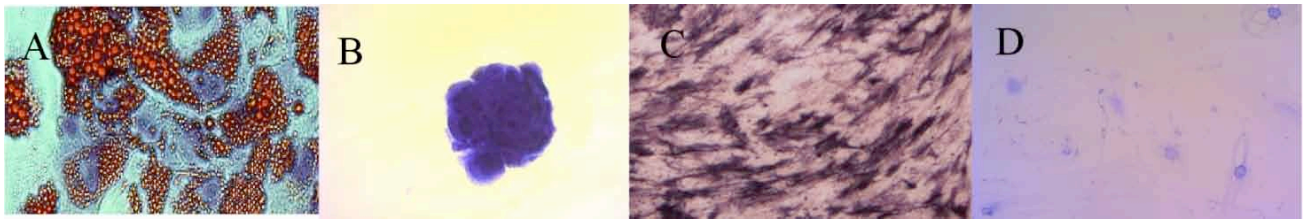

Fig. (8). Multilineage differentiation of MSCs. (**A**) Adipogenic differentiation. MSCs are capable of forming Oil Red O positive cells. Adipogenic-induced cells contained single lipid droplets, stained orange by oil red O. (**B**) Osteogenic differentiation was demonstrated by the presence of mineralized nodules stained black/purple with von Kossa staining. b-glycerophosphate significantly increased formation of mineralized nodules. (**C**) Chondrogenic differentiation. Chondrogenic differentiation was assessed histologically by demonstrating the presence of cartilage-related matrix components in the specimens. Chondrogenic-induced pellets showed intense purple metachromasia in toluidine blue staining, indicating a high content of sulfated proteoglycans. (**D**) Fibroblast differentiation. The presence of fibroblasts in culture was confirmed by staining blue by May-Grunwald. Original magnification, × 400.

factors were differentiated into mature adipocytes, fibroblasts, osteoblasts and chondrocytes. This is confirmed by histological studies (Fig. **8**).

Adipogenic Differentiation

MSCs were grown in adipogenic medium with indomethacin and insulin. Cells from the histologically normal mice and the mice with fibrosis, treated with NaCl, accumulated small lipid droplets (Fig. **8**, **A**). Mergers of lipid droplets in the large vacuoles are not registered. There were not revealed significant differences in the number of cells with lipophilic inclusions in samples, obtained from healthy mice and and from mice with fibrosis and treated with NaCl. Reserpine prevented the accumulation of lipid droplets in cells of fibrotic lung. Proportion of cells, containing lipophilic inclusions, was 3.00 ± 0.48% of the total number of cultured cells in the experimental group, 11.00 ± 2.32% (n = 8, p <0.001) - in cell samples of healthy mice, 10.00 ± 1.85 % (n = 8, p <0.001) - in samples of fibrotic cells of mice with NaCl.

These results indicate that the final differentiation of pulmonary MSCs of all the investigated sources in mature adipocytes was not achieved. Reserpine significantly reduced the activity of adipogenesis.

Chondrogenic Differentiation in Three-Dimensional Granular Cultures

MSCs from all studied sources formed the sedimentary (granular) culture in chondrogenic medium with TGF-β_1. Histological examination showed the presence of cartilage and associated components of the matrix in the samples. Toluidine blue staining granules detected intense violet metachromasia, indicating high levels of sulfated proteoglycans (Fig. **8, B**). Staining by toluidine blue showed a high content of collagen fibers in the chondrogenic induced granular cultures. However, cells in the pellets were collected in friable conglomerate of several structural components of the extracellular matrix in samples obtained from fibrotic mice with reserpine. Proportion of cells with colored acid and acetate mucins in histological samples was lower (34.00 ± 4.12%), than in cultured cells of healthy mice (62.00 ± 7.21%, (n = 8, p <0.01)) and in cultured cells of mice with fibrotic NaCl (67.00 ± 7.82%, (n = 8, p <0.01)).

Thus, treatment of reserpine significantly reduced the intensity of chondrogenic differentiation of MSCs of fibrotic lung.

Osteogenic Differentiation

Clustering is a characteristic feature of osteogenic differentiation of mesenchymal stem cells *in vitro* [18]. In our studies, MSCs of histologically healthy mice formed separate clusters of cells, growing in multiple layers in osteogenic medium with β-glycerophosphate. Positive staining by von Kossa and alizarin red S cells confirmed the deposition of calcium and phosphate in the extracellular matrix. Area of (S) deposition of calcium was 136.13 ± 24.03 mm^2 (Fig. **8**, **C**). MSCs of fibrotic mice treated with NaCl, had a lower ability to form clusters in osteogenic medium with β-glycerophosphate compared to cells of healthy mice (S = 29.32 ± 3.11 mm^2, (n = 8, p <0.001)). Cells formed several separate clusters, instead of merging cultures. Most cells of fibrotic mice with reserpine retained in cultures typical morphology of fibroblasts and their characteristics of growth with minimum friable mineralized nodules in the extracellular matrix. Area of mineralization was 6.45 ± 0.81 mm^2 and this was significantly lower to that of histologically healthy mice and fibrotic mice treated with NaCl.

This observation demonstrated a violation of extracellular mineralization of bleo-treated MSCs *in vitro*. Reserpine exacerbated the inhibition of osteogenesis.

Fibroblast Differentiation

Fig. (**8D**) shows fusiform cell morphology from all the samples in the fibroblastic medium with fibroblast growth factor. Staining by the basic dye May-Grunwald confirmed the presence of fibroblasts in culture. The number of fibroblasts in the culture of cells, derived from fibrotic mice, treated with NaCl was at 29.57% (n = 8, p <0.05) more than in the cell culture of healthy animals. Reserpine reduced the amount of fibroblasts in samples from bleo-treated mice to the level of healthy animals.

Thus, reserpine had an inhibitory effect on fibroblast differentiation of MSCs from bleomycin treated lungs.

The Study of Cell Colonies in Culture

Undifferentiated Hematopoietic Colony-Forming Units (CFU-U)

Fig. (**9**) A illustrates the increase of the CFU-U growth intensity in liquid cultures of non-adherent bone marrow cells of mice with pneumofibrosis, treated with NaCl, compared to samples of non-adherent bone marrow cells of

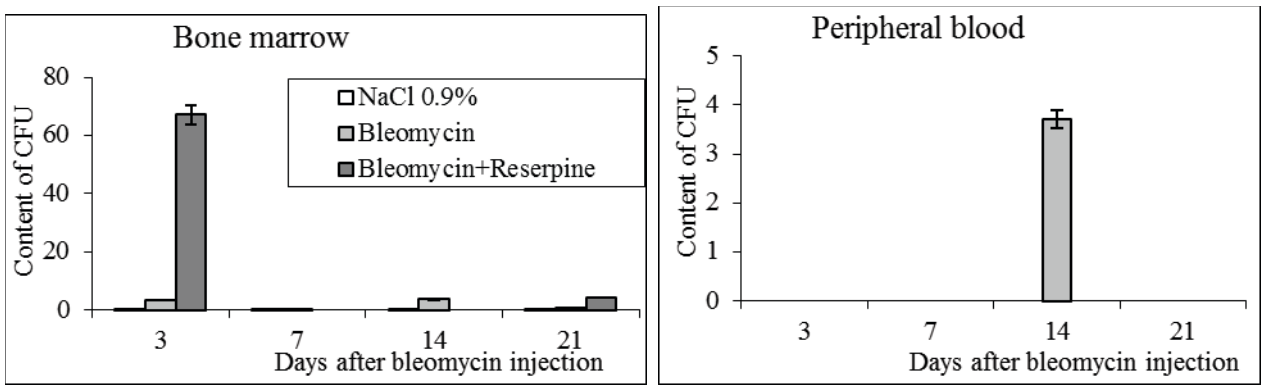

Fig. (9). The effect of reserpine on content of CFU-U in bone marrow and peripheral blood of C57BL/6 mice with pulmonary fibrosis. Mice received intratracheal of NaCl 0.9% (**NaCl 0.9%**), mice received intratracheal of bleomycin (**Bleomycin**), mice received reserpine after intratracheal administration of bleomycin (**Bleomycin+Reserpine**). The number of colonies was showed on Y-line. CFU-U was counted using inverted microscope (> = 500 cells per colony), morphological analysis of colonies was carried out. Data represent mean colony counts ± SEM. Assays were performed in duplicate.

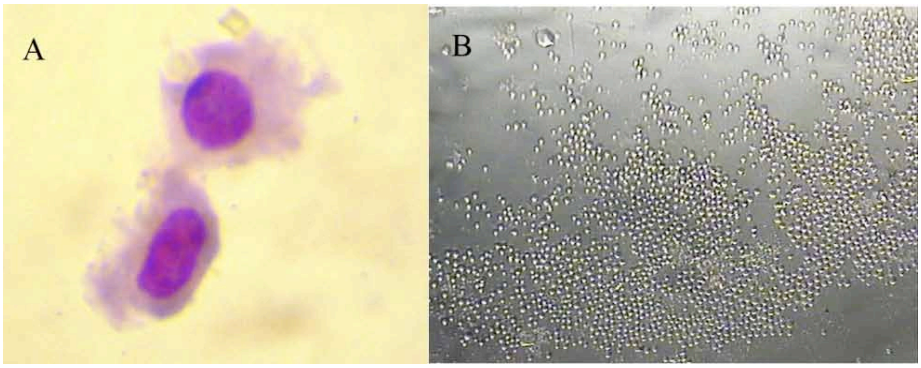

Fig. (10). (**A**) CFU-U, stained with May-Grunwald. (**B**) Reverse phase contrast image of CFU-U.

histologically healthy mice on the 3rd, 14th days experience. In cultures of non-adherent blood cells of fibrotic mice CFU-U appeared on the 14th day.

Reserpine treatment leads to significantly increasing (n = 8, p <0.0001) growth of CFU-U intensity in the culture of bone marrow cells from bleo-treated mice on the 3rd day of the experiment. On 14th day colonies in the samples as in group of mice with fibrotic NaCl are not found (Fig. **9**). Recovery of activity colony formation is observed on 21th experiment day. Reserpine has a suppressive effect on the formation of CFU-U in non-adherent blood cell cultures (14th day).

Morphologic analysis with staining by May-Grunwald found, that the cells forming CFU-U, contained the majority of morphologically undifferentiated nuclears and did not exceed the size of lymphocytes (Fig. **10**).

We studied the immune profile of CFU-U cells. It was shown that the cells of the samples (blood, bone marrow) in the control and experimental groups were negative for markers of mature hematopoietic cell populations ($CD3^-$, $CD45R^-$ (B220), $Ly6C^-$and $Ly6G^-$ (Gr1), $CD11b^-$, TER^- 119^-), positive for the markers Sca-1, c-Kit and negative for CD34 marker.

The results showed that reserpine reduced the clonal HSCs activity from blood of fibrosis mice. Suppression of CFU-U in the culture of bone marrow was noted after their "explosive" growth on the 3rd day of the experiment.

The Mixed (Granulocyte - Erythroid - Macrophage - Megakaryocytic) Colony Forming Units (CFU-GEMM)

Yield of CFU-GEMM in liquid cultures of non-adherent bone marrow cells from fibrotic mice, treated with NaCl, increased more than 4 times (n = 8, p <0.01) in compared to samples of healthy animals on the 3rd day of the experiment (Fig. **11**). The appearance of CFU-GEMM in cultures of non-adherent peripheral blood cells was observed on the 3rd day, on the 7th day. The number of colonies in the samples reached their maximum values $2.33 \pm 0.25 \times 10^5$ non-adherent cells.

Reserpine had a strong suppressive effect on the ability of non-adherent bone marrow and blood cells to form CFU-GEMM (Fig. **11**). Thus, the suppressive effect was observed on the 3rd and 7th days of the experiment.

The presented results show that reserpine had suppressive effect on the CFU-GEMM growth in samples of blood and bone marrow, derived from cells of mice with fibrosis.

Granulocyte Colony-Forming Units (CFU-G)

The research of methylcellulose cultures of non-adherent bone marrow cells from fibrotic mice, treated with NaCl, showed the CFU-G growth throughout the study. The maximum intensity of colony formation is observed on the 3rd day of the experiment (Fig. **12**). The activity of colony formation in the blood cell cultures of fibrotic mice was increased on the 21st day. During this period, the number of CFU-G in samples was $6.53 \pm 0.75 \times 10^5$ non-adherent cells

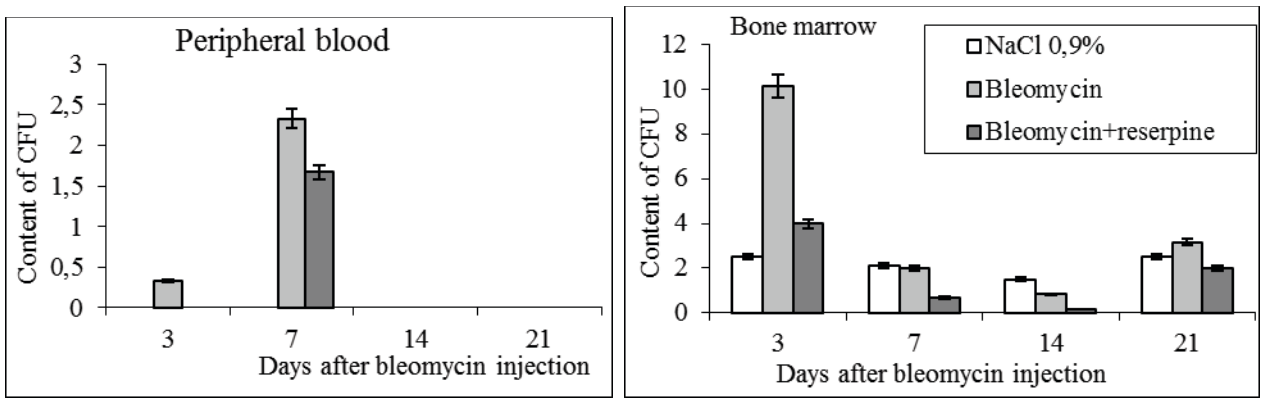

Fig. (11). The effect of reserpine on content of CFU-GEMM in bone marrow and peripheral blood of C57BL/6 mice with pulmonary fibrosis. Mice received intratracheal of NaCl 0.9% (**NaCl 0.9%**), mice received intratracheal of bleomycin (**Bleomycin**), mice received reserpine after intratracheal administration of bleomycin (**Bleomycin+reserpine**). The number of colonies was showed on Y-line. CFU-GEMM was counted using inverted microscope (> = 500 cells per colony), morphological analysis of colonies was carried out. Data represent mean colony counts ± SEM. Assays were performed in duplicate.

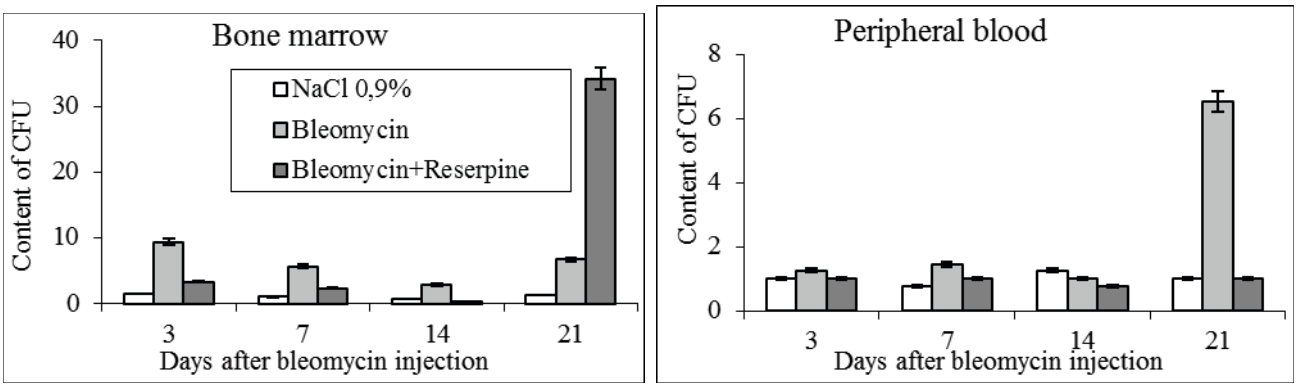

Fig. (12). The effect of reserpine on content of CFU-G in bone marrow and peripheral blood of C57BL/6 mice with pulmonary fibrosis. Mice received intratracheal of NaCl 0.9% (**NaCl 0.9%**), mice received intratracheal of bleomycin (**Bleomycin**), mice received sympatholytic after intratracheal administration of bleomycin (**Bleomycin+Reserpine**). The number of colonies was showed on Y-line. CFU-G was counted using inverted microscope, morphological analysis of colonies was carried out. Data represent mean colony counts ± SEM. Assays were performed in duplicate.

(n = 8, p <0.01) at 1.00 ± 0.48 × 10^5 non-adherent cells at histologically healthy mice (n = 8).

Reserpine treatment reduced the yield of CFU-G in the culture to 1.00 ± 0.28 × 10^5 non-adherent cells (21st day) (Fig. **12**). Reserpine continuously reduced the colonies growth rate in the cultures of bone marrow (3rd, 7th, 14th days). It is noteworthy that to the 21st day of the experiment the number of CFU-G significantly increased to 34.17 ± 4.45 × 10^5 non-adherent cells (n = 8, p <0.001) at 6.67 ± 0.72 × 10^5 non-adherent cells in group of fibrotic mice with NaCl (n = 8).

Thus, the injection of reserpine reduced clonal activity of granulocytic precursors of blood and bone marrow at fibrotic mice. The cancel of reserpine led to "explosive" growth of CFU-G in bone marrow culture.

Fibroblast Colony-Forming Units (CFU-F)

Analysis of CFU-F showed, that liquid cultures of adherent mononuclear cells from fibrotic lungs of mice generated single fibroblast colony already on the 3rd day of experience. Further gradual increase in the activity of CFU-F formation was noted: their number was 3.00 ± 0.40 × 10^5 cells on the 7th day of the experiment, 11.00 ± 0.80 × 10^5 cells on 14th day, 18.30 ± 1.70 × 10^5 cells on 21st day. It should be noted that throughout the observation period in the cultures of adherent cells from histologically healthy lung colonies was not identified. Stimulation of the CFU-F growth was observed in cultures of adherent bone marrow cells and adherent cells of peripheral blood (Fig. **13**). The highest intensity of fibroblast colony formation in bone marrow samples was noted on the 3rd and 7th days of the experiment, in peripheral blood samples on the 21st day. In cultures of adherent mononuclear of fibrotic lungs of mice treated with reserpine, CFU-F on the third day of the trial was identified, on the 7th and 14th day detected single colony, on the 21st day their number was 8.27 ± 1.28 × 10^5 adherent mononuclear cells of the lungs.

However, the response of fibroblast progenitor cells in the bone marrow and peripheral blood to the introduction of reserpine was not so clear. Initially, the samples of bone marrow (third day) and peripheral blood (3rd, 7th day) recorded an increase in the CFU-F growth (Fig. **13**). Decrease of process activity under reserpine treatment was observed on the 7th, 14th and 21st days experience in marrowy cultures, and on the 21st day - in cultures of peripheral blood.

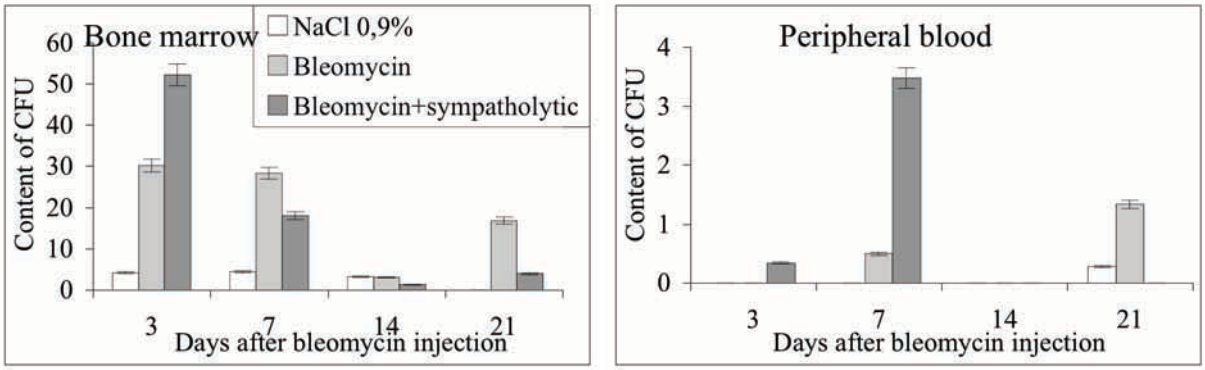

Fig. (13). The content of CFU-F in culture bone marrow derived and peripheral blood derived cells from C57BL/6 mice with pulmonary fibrosis. Mice received intratracheal of NaCl 0.9% (**NaCl 0.9%**), mice received intratracheal of bleomycin (**Bleomycin**), mice received sympatholytic after intratracheal administration of bleomycin (**Bleomycin+sympatholytic**). The number of colonies was showed on Y-line. The CFU-F (> = 50 cells per colony) were counted using an inverted microscope and morphological analysis of colonies was carried out. Data represent mean colony counts ± SEM. Assays were performed in duplicate.

These results suggest that reserpine had a suppressive effect on the activity of the clonal progenitor of fibrotic lung fibroblast cells. It is noteworthy that the reduction in the growth CFU-F in the cultures of bone marrow and blood is preceded by a short-term stimulation of the process.

DISCUSSION

These studies allowed us to make a few important conclusions. First, an increase of neutrophils and lymphocytes in bone marrow and blood (7^{th}, 14^{th} days) was before acute inflammation in the lungs (with the assistance of macrophages, lymphocytes, neutrophils, plasma cells in the parenchyma), induced by bleomycin IT administration. Monocytes also significantly raised in the tissues (data not shown). Second, the increase of clonal activity of HSCs and hematopoietic progenitor cells (CFU-GEMM, CFU-G) from bone marrow is preceded chronologically. Clonal activity is observed on the part of HSCs and progenitor cells of the blood. There were study points to the migration of hematopoietic cells by the bloodstream to organs (including the lungs) [19]. Thus hematopoietic cells might be involved in the processes of repair and regeneration. The results of this study support this hypothesis. Most likely, the mobilization of bone marrow HSCs and hematopoietic progenitor cells occurs at the bleomycin-induced fibrosis. They migrate into the lung parenchyma with their preliminary differentiation into mature inflammatory cells (particularly neutrophils and monocytes). Drop in c-Kit expression on the surface of bone marrow HSCs can mean their entry into the differentiation. This is supported by our recent studies where the formation of predominantly CFU-F was shown, as well as macrophage and granulocyte-macrophage colony-forming units in the culture of bone marrow HSCs, derived from C57BL / 6 mice with fibrosis (data not shown). Thus, the selective differentiation of HSCs in granulocyte progenitor cells can be explained by rapid formation of CFU-F in bone marrow culture throughout the study. The question of possible further differentiation of progenitor granulocyte-macrophage, granulocyte and macrophage cells to monocytes, neutrophils, for the presents is open. The answer needs additional researches.

Fibroblasts are the main source of interstitial collagen. Due to vigorous replication of fibroblasts and excessive deposition of extracellular matrix, occurs the destruction of the airspace of the lungs. It is believed that the origin of fibroblasts is in lung. However, there are indications of circulating blood cells (called "fibrocytes"), which have the fibroblast-like properties and are able to migrate to the damaged tissue [20]. It is believed that circulating fibroblast cells "settle" in the inflammation area in the lungs and are involved in the development of fibrotic changes [21, 22]. Hashimoto N. *et al.* (2004) in their studies injected bone marrow cells from transgenic mice expressing GFP to adult mice. Intratracheal administration of bleomycin led to increase of the number of GFP $^{+}$ cells in fibrotic lung at chimeric mice at the same time a significant increase in GFP $^{+}$-cells expressing type I collagen was detected [23]. According to our information, the installation of bleomycin in mice C57BL/6 causes the increase in the activity of clonal primarily fibroblast progenitor cells in the bone marrow (3^{rd}, 7^{th} day), then fibroblast progenitor blood cells (7^{th}, 21^{st} days) and pulmonary cells (7^{th}, 14^{th}, 21^{st} days). As seen, our studies support the hypothesis that fibrotic lung fibroblast cells may be originate from bone marrow. We should not forget about the MSCs participation in fibrotic changes in the lung. This idea is supported by evidence that MSCs are able to migrate to damaged tissues [3, 24]. *In vitro* it is shown that MSCs at pulmonary fibrosis selectively differentiate into fibroblast cells [17]. These arguments provide a basis for the revision of understanding not only the fibroblasts origin in the lungs, but pneumofibrosis pathogenesis and treatment of pulmonary fibrosis.

In this study, sympatholytic reserpine significantly reduces the intensity of destructive processes in the lungs of bleo-treated mice: reduces infiltration of alveoli and alveolar passages by inflammatory cells (lymphocytes, macrophages, neutrophils, plasma cells), and prevents the growth of connective tissue in the lungs. Reserpine simultaneously cancels the neutrophilic leukocytosis and elevates levels of lymphocytes in the blood. Similarly, reserpine affected bone marrow cells: reduced the number of neutrophilic granulocytes, monocytes and lymphocytes to the level of histologically healthy mice. Taking into account the hypothesis that bone marrow HSCs, MSCs and progenitor cells are involved in the development of fibrosis, we evaluated the possible mechanisms of reserpine action.

These experiments showed that the sympatholytic activity reduced clonal bone marrow and granulocytic precursors (3^{rd}, 7^{th}, 14^{th} days). At the same time reserpine reduced the growth rate of CFU-GEMM and CFU-G hematopoietic progenitor cells from circulating blood.

Our results showed that reserpine treatment significantly increased clonal activity of bone marrow cells with the phenotype of Lin-, Sca-1^+, c-Kit^+, $CD34^-$ (3^{rd} day). Then the accumulation of HSCs in the bone marrow and in the fibrotic lung is followed (7^{th} day). A possible explanation of this we found in the hypothesis of 'niches' for HSCs. Postnatal HSCs in the bone marrow are in equilibrium with a small fraction of the circulating blood. During mobilization (for various reasons - the introduction of drugs, illness) HSCs, presumably, have to leave the "microenvironment" in the bone marrow to migrate through the endothelial barrier and circulate in the bloodstream without immediate return (homing) to the bone marrow or other organ [Wilson, ATA, 2006][25]. It was thought that HSCs are located in the immediate vicinity of the upper layer of the bone marrow in contact with osteoblasts, lining the transition of cortical bone marrow (endosteum) [26, 27]. Another suggested location in the bone marrow for HSCs vascular 'niche', where HSCs can be attached to the fenestrated endothelium of specialized bone marrow vessels, "blind bags", called sinusoids [28]. Sinusoids of the bone marrow produce molecules that are important for the mobilization of HSCs, and homing, and living, including cytokines such as CXCL12 and adhesion molecules, endothelial cell E-selectin and vascular cell adhesion molecule -1 (VCAM-1). Such a view is supported by the findings that HSCs with a profound defect in cell migration is the result of a combined loss of function of signaling pathways CXCRL4, β1-integrin and c-kit - can be mobilized in large numbers [29]. The sympathetic nerve fibers are elements of 'niche' [30]. Osteoblasts express β_2-adrenergic receptors, thus becoming the direct effectors of sympathetic transmission of signals, which in some cases helps to restore the bone marrow [31]. Meanwhile, sympathetic neurons do not establish synapses with every cell [30]. Presumably, the increased of signal synchronization to neighboring cells happens through direct cell-cell communication system. Thus, adrenergic system may participate in the management of a sensitive balance between the pool of bone marrow HSCs and peripheral blood HSCs. Reserpine inhibits vesicular uptake of catecholamines and serotonin. It should be noted that reserpine has a biphasic effect on the tonic and reflex activity of the sympathetic nervous system, as well as on vasomotor reflexes [32-34]. The first depriming phase of the reserpine action on the regulation of vasomotor centers is due to excitation of the released monoamines central monoaminergic inhibitory mechanisms. This phase coincides with an increase of free, functionally active form of monoamines in the brain. The second phase of action after administration develops after 2-4 hours and is characterized by the increase in tonic activity and vasomotor reflexes and complete elimination of the inhibition reflex in the sympathetic nerves. The second phase will coincide with a period of stock depletion labile monoamines in the brain. The clinic of hypertensive patients with reserpine administration had an initial increase in blood pressure, leading to cessation of therapy. From our point of view, short-term elevated levels of free and functionally active form of monoamine can be explained by HSCs yield from the "microenvironment" in terms of reserpine administration. It is noteworthy that effect of reserpine "explosive" growth of clonal hematopoietic stem cell activity was detected by us in the bone marrow of mice, damaged by cyclophosphamide [11]. As can be seen, the reaction of HSCs to the reserpine introduction is independent of modulating disease.

Picture of the anti-inflammatory action of reserpine complements the fact that differentiation of bone marrow HSCs in the granulocyte-macrophage progenitor, granulocyte and macrophage cells is not followed under the reserpine treatment (from recent experimental studies have not shown). The absence of HSCs in the blood, with a certain probability, may indicate a violation of migration through the endothelial barrier and circulation in the bloodstream. Lung HSCs can not be ignored, because, in our view, they are the subpopulation of cells, migrating from the bone marrow. However, we have not explained the rise in lung cells with the phenotype Lin , Sca-1^+, c-Kit^+, $CD34^-$, at decreasing indications of inflammation (reduction in the number of macrophages, neutrophils in the parenchyma) (7^{th} day) at the injection of reserpine.

Thus, we believe that the inflammation in bleo-treated lungs may be reduced by the reserpine due to the inhibitory effect on bone marrow HSCs and progenitor hematopoietic cells (3^{rd} – 14^{th} days). Violation of hematopoietic cells migration in the bone marrow under the influence of reserpine remained in terms of the most active deposition of fibrotic mass in the lungs on the 21^{st} day. This conclusion was based on the evidence of increasing pool of bone marrow cells expressing pan-hematopoietic marker CD45, and significantly increased their clonal activity (formation of CFU-F). At the same time, histology of fibrotic lungs, level of HSCs from fibrotic lung and bone marrow became normal, clonal activity of $CD45^+$-blood cells was canceled.

Part of mesenchymal origin cells in the pneumofibrosis development directed our interest to study the reserpine effect on MSCs. According to our data, reserpine reduced the population of bone marrow CD45-cells in time of intensive collagen deposition in the lung (21^{st} day), thus the number of cells showing mesenchymal phenotype ($CD44^+$, $CD73^+$, $CD90^+$, $CD106^+$, $CD31^-$, $CD34^-$, $CD45^-$) was reduced. The number of MSCs in fibrotic lung parenchyma significantly reduced. MSCs decline from lungs developed simultaneously with the reduction of their differentiation into fibroblast-like cells. It is noteworthy that on the 21^{st} day of the experiment, a similar reaction to reserpine was observed by fibroblast progenitor cells in the bone marrow, blood, and lungs (CFU-F). Meanwhile, clonal progenitor activity of fibroblast cells in the bone marrow and in the blood increased in the active phase of inflammation (3^{rd}, 7^{th} days) and the CFU-F in fibrotic lung missing. We believe that improvmet of histological characteristics under the reserpine may be due to a violation of bone marrow-derived fibroblast progenitor cells migration into the lung tissues damaged by bleomycin. Another important aspect of the anti-fibrotic activity of reserpine is possibly related to epithelial cells. Presumably MSCs-like cells are attracted to the fibrotic lung, differentiated into the epithelial cells of the lungs, thereby improving fibrosis [35]. It is believed that stem cells are

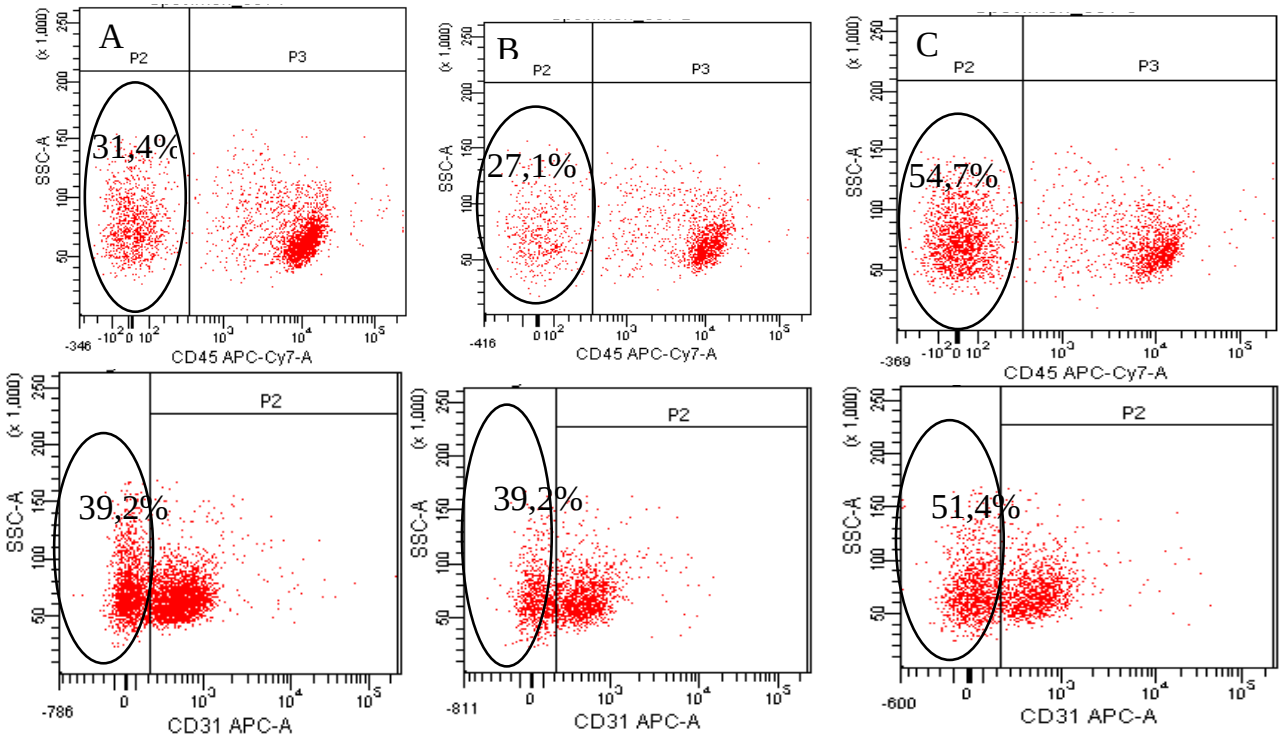

Fig. (14). Flow cytometric analysis of cell-surface antigens on murine mesenchymal stem cells from lungs, derived on the 21st day of experiment. The figure plots show the number of CD45- cells (percentage of labeled cells) and the number of CD31- cells (percentage of labeled cells). (**A**) mice with intratracheal administration of NaCl 0.9%, (**B**) mice with intratracheal administration of bleomycin, (**C**) mice with intratracheal administration of bleomycin and treated with reserpine. A, B and C percentages represent the mean from 3 different samples

defined as bronchioles CD45neg, CD31neg, CD34neg, SCA-1^{low} and AF^{low} [36]. Our cytometric studies showd that reserpine increases the concentration of lung cells, which are defined as CD45neg, CD31neg, and CD34neg (Fig. **14**).

As it is seen, the situation with the differentiation of MSCs, involved in damaged lungs, is rather ambiguous. Thus, the reduction of bleomycin-induced pulmonary fibrosis after reserpine treatment may be associated with a decrease in the migration of bone marrow MSCs and progenitor fibroblast cells into the fibrotic lungs. It should be noted that reserpine, possibly, has an inhibitory effect on the differentiation of MSCs, involved into the lungs, in fibrocytes, while not excluding their differentiation into epithelial cells.

It is believed that the use of stem cells as a transplant is the most promising approach in the treatment of degenerative diseases, including fibrotic lung disease. However, stem cell therapy is accompanied by an increased risk of bacterial and viral infections, allergic reactions [37, 38]. Serious obstacle in particular MSC-therapy is the difficulty to obtain sufficient numbers of cells for transplantation, and the lack of standardized and effective methods to obtain stem cells [39]. Previous studies demonstrated a decrease in damaged lung tissue and collagen deposition in mice with bleo-induced fibrosis, treated with antiserotonin drug cyproheptadine [40, 41] and neuroleptic haloperidol [11, 42]. The results of our recent studies indicate the involvement of mesenchymal and hematopoietic stem cells in the anti-fibrotic effect of antiserotonin drug and neuroleptic. Thus, we believe that the modulation functions of endogenous stem cells by neural-pharmacological agents may be a promising approach in the treatment of fibrotic lung disease.

CONCLUSIONS

Initiated by IT administration of bleomycin inflammation in lungs of mice C57BL/6 is accompanied by an increase of bone marrow, circulating in the blood and lung HSCs. In this case, an increase in the clonal activity of hematopoietic progenitor cells in the bone marrow and blood is observed, as well as selective differentiation of HSCs in granulocyte progenitor cells. Simultaneously with the deposition of collagen fibers in the interstitial tissue electoral differentiation of lung MSCs into fibroblast-like cells is registered, the formation of CFU-F from progenitor fibroblast cells in the bone marrow, blood and lungs is determined.

It is shown that reserpine reduces infiltration of alveoli and alveolar interstitium passages by inflammatory cells, prevents the growth of connective tissue in the lung parenchyma. Anti-inflammatory effect of reserpine is accompanied by the decrease in the differentiation activity of bone marrow HSCs in CFU-G, the decrease of circulating HSCs and hematopoietic progenitor cells, and impaired cell migration into the damaged by bleomycin lungs. Retardment fibrosing of interstitial lung tissue by reserpine was associated with a reduction of MSCs and progenitor subpopulations of fibroblast cells in the lungs, and with violation of MSCs differentiation into fibroblast-like cells.

MATERIALS AND METHODS

Animals

C57BL/6 mice, in the amount of 455, 8-10 weeks of age, were purchased from the Dept. of Biomodels, Research Institute of Pharmacology, SB of RAMS (Tomsk, Quality Certificate Number 188-05). Mice were housed 5 animals per cage (VELAZ) under normal laboratory conditions, i.e. room temperature (22±2° C), relative humidity of 55±10%, 12/12 h light-dark cycle. Food and water were freely available. All experimental procedures with animals were performed in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (March, 18, 1986, Strasburg, ETS 123) and the Order of Public Health Ministry of Russian Federation 267 (Moscow, 2003, June, 19). All animal experiments were approved by the Animal Care Committee of the Research Institute of Pharmacology.

Reagents

Bleomycin sulphate was purchased from Bristol Myers-Squibb (Blenoxane®, São Paulo, Brazil).

Reserpine Experimental Design and Bleomycin Lung Injury

Bleomycin was injected intratracheally 80 ug / mouse in 30 ml NaCl 0.9% [5]. Control animals were injected intratracheally 30 ml NaCl 0.9%. At the 1, 2, 3, 4, 5, 6, 7, 9-th day after bleo-injection mice were injected intraperitoneal sympatholytic reserpine ("SIGMA", USA) in dose 1 mg/kg in 100 ul NaCl 0.9%. Mice were sacrificed by an overdose of CO_2 for 3, 7, 14, 21, 25, 40, 60 days after bleomycin administration.

Histopathological Studies of Lungs

Lungs were collected and immediately fixed in 10% formalin. Fixed specimens were dehydrated in alcohols of increasing concentrations. Specimens were processed, embedded in paraffin and cut into 4-5 μm sections and stained with haematoxylin and eosin (H&E). For evaluation of collagen fibres in the pulmonary parenchyma, the slides were stained with picrofuchsin [43]. 10 photomicrographs of lung tissue were made over the whole area of cut at 400× without overlap for each experimental animal. The system used consisted of a video camera (AxioCam ERc5s, Carl Zeiss, Göttingen, Germany), applied to a microscope (Axio Lab.A1, Carl Zeiss MicroImaging GmbH, Göttingen, Germany) attached to a computer. The images were processed by software AxioVision Rel.4.8.2. Content of collagen fibers in the lungs was determined by using a special function to determine the area of the object in the picture. Bronchovascular bundles were carefully avoided during the measurements. The means was calculated and the values expressed as a percentage [44, 45].

Morphological Examination of the Blood and Bone Marrow

Blood: The study of the total number of white blood cells, granulocytes and lymphocytes was conducted by standard hematological methods [46]. Fixation blood preparations were made by May-Grunwald for 5-7 minutes. Staining was performed according to the method Nocht-Maksimov for 25 minutes (with azure II-eosin). Hemogram was counted on 100 cells, and then was determined the absolute content of granulocytes and lymphocytes in the blood. Reticulocytes - erythrocytes containing granular-filamentous substance (subsantia granulo-filamentosa), were identified by supravital staining brillyantkrezilblau.

Bone marrow: To study the bone marrow cellularity mouse femur was isolated, and was purified from the soft tissues. Then central channel was washed with 1 ml of 3% acetic acid. The bone marrow was resuspended with syringe needle through decreasing diameter. The total number of myelokaryocytes was counted in Goryaev chamber. For the preparation of smears of of bone marrow femur contents were squeezed to skim glass, then were diluted with autologous serum and smeared with ground glass. Fixation of bone marrow preparations was made by May-Grunwald for 3-5 minutes. Staining was performed according to the method Nocht-Maksimov for 20 minutes (with azure II-eosin). Myelogram was counted in 400 cells, and then were determined the absolute contents of immature and mature neutrophilic granulocytes, lymphocytes and erithrocariocytes in bone marrow.

Flow Cytometric Analysis

Membrane receptor's expression of murine MSCs from bone marrow or lung was assayed according to the protocol (BD Biosciences, CIIIA). The cells were divided into aliquots of 200 000 in 1.5-mL centrifuge tubes and incubated at 4 C for 30 minutes with the following antibodies: anti-rat CD90 (Thy-1)/mouse CD90.1 (PerCP), CD34 FITS, CD45 PE/Cy5, CD 73 (PE), CD106 (VCAM-1) FITS, CD44 (Pgp-1, Ly-24) APC and anti-mouse CD31 (PECAM-1) APC (BD Biosciences, US). Next isotype controls were used in this study: FITS Rat IgG2a, PerCP Mouse IgG1, APC Rat IgG2b, APC-CyTM 7 Rat IgG2b, PE Rat IgG2a. Then the cells were pelleted by centrifugation for 1 minute at 450g. Supernatant was removed. The cells were resuspended in 1 mL PBS. The cells were pelleted, washed twice with PBS, and analyzed by flow cytometry. A minimum of 10,000 events were recorded for each tube, using a FACSCanto II flow cytometer, and analyzed with cell quest software (BD Biosciences, US).

Membrane receptor's expression of murine HSCs from bone marrow or lung was assayed according to the protocol for BD Mouse Hematopoietic Stem and Progenitor Cell Isolation Kit (BD Biosciences, US). The cells were divided into aliquots of 200 000 in 1.5-mL centrifuge tubes and incubated at 4 C for 30 minutes with the following antibodies: APC Mouse Lineage Antibody Cocktail (CD3, CD45R (B220), Ly6C and Ly6G (Gr1), CD11b (Mac1), TER-119) (BD Pharmingen™), PE-Cy™ 7 Anti-Mouse Sca-1 (BD Pharmingen™), PE Anti-Mouse c-Kit (BD Pharmingen™), FITS Anti-Mouse CD34 (BD Pharmingen™). Then the cells were pelleted by centrifugation for 1 minute at 450g. Supernatant was removed. The cells were resuspended in 1 mL PBS. The cells were pelleted, washed twice with PBS, and analyzed by flow cytometry. The 10,000 events minimum were recorded for each tube, using a FACSCanto II flow cytometer, and analyzed with cell quest software (BD Biosciences, US).

Cultural Studies

The Cloning of Undifferentiated Hematopoietic Colony-Forming Units

Cultures were performed in 96-well plates ("Costar", USA) with 5×10^5 non-adherent bone marrow cells or blood / 1 ml basic culture medium based on Dulbecco's modified Eagle's medium (D-MEM, «Sigma», USA) 25% fetal calf serum, oh ("Bioclot", Germany), 1% bovine serum albumin (V-cut) ("Sigma", USA), 10-5 M 2-mercaptoethanol ("Sigma", USA), erythropoietin 2 U / mL ("Sigma", USA), 4 ng / ml interleukin-3 ("Sigma", USA), 3 ng / ml of stem cell growth factor (factor Steele) ("Sigma", USA), 10 ng / ml insulin-like growth factor-1 ("Sigma", USA), 280 mg / l L-glutamine ("Sigma", USA), 100 U / ml penicillin ("Sigma", USA), 100 mg / ml streptomycin ("Sigma" , USA), 10-6 dexamethasone ("Sigma", USA). Incubation was performed at 37 ° C and 5% CO2 in the atmosphere, absolute humidity, on the 7th day was carefully removed to 50% of the supernatant and was added fresh basic culture medium of the same volume. At the end of the 14th day of incubation, the cells of the primary culture were collected, washed twice with D-MEM and to some basic culture mediums were made in 96-well plates and incubated under certain conditions. Non-adherent concentration of cells during passages of 5×10^5 cells / mL in 1 mL basic culture medium: Passivation was performed twice. At the end of the last cycle of cultivation CFU-U was counted using an inverting microscope (≥ 500 cells in the colony), and then was conducted morphological analysis of colonies studied the immune profile of cells that make up the CFU-U [46, 47].

The cloning of granulocyte - erythroid - macrophage - megakaryocyte colony-forming units

Cultures were performed in 24-well plates ("Costar", USA) with 1×10^5 non-adherent bone marrow cells or blood / 1 ml basic culture medium based on Dulbecco's modified Eagle's medium (D-MEM, «Sigma », USA) with 20% fetal calf serum, oh (" Bioclot ", Germany), erythropoietin, 2 U / mL (" Sigma ", USA), 2 ng / ml IL-3 (" Sigma ", USA), 0.2 ng / ml granulocyte-macrophage colony stimulating factor ("Sigma", USA), 10-5 M 2-mercaptoethanol ("Sigma", USA), 1% bovine serum albumin ("Sigma", USA), 280 mg / l l-glutamine ("Sigma", USA), 50 mg / L gentamicin ("Serva", Germany). Incubation was carried out at 37° C and 5% CO2 in the atmosphere, the absolute humidity, on the 7th day was carefully removed to 50% of the supernatant and was added fresh basic culture medium of the same volume. At the end of the 12th day of incubation the CFU-GEMM was counted (≥ 500 cells in the colony) using an inverting microscope and was conducted morphological analysis of colonies [46, 47].

The Cloning of Granulocyte Colony-Forming Units

Cultures were performed in 24-well plates (15 mm diameter cup) ("Costar", USA) with 2 × 105 cells non-adherent blood or bone marrow / 1 ml of culture medium base based on RPMI-1640 ("Sigma", USA) with 20% fetal calf serum, oh ("Bioclot", Germany), 280 mg / l l-glutamine ("Sigma", USA), 50 mg / L gentamicin ("Serva", Germany), 10-5 M 2 - mercaptoethanol ("Sigma", USA), granulocyte colony-stimulating factor of 4 ng / mL ("Sigma", USA) and 0.9% solution of methyl cellulose ("Sigma", USA). Incubation was carried out at 37 ° C and 5% CO2 in the atmosphere, the absolute humidity. CFU-G was counted on the 7^{th} day (≥ 50 cells in the colony), using an inverting microscope and then was conducted morphological analysis of colonies [46].

The Cloning of Fibroblast Colony-Forming Units (CFU-F)

Cultures were performed in 24-well plates with 1×10^5 cells adhering bone marrow, blood or lungs / 1 ml of culture medium base by D-MEM with 10% fetal calf serum ("Bioclot", Germany), 280 mg / l l-glutamine ("Sigma", USA), 50 mg / L gentamicin ("Serva", Germany), 25 ng / ml fibroblast growth factor (FGF-basic) ("Sigma", USA), 10-6 dexamethasone ("Sigma", USA). Incubation was carried out at 37 ° C and 5% CO_2 in the atmosphere, the absolute humidity for 10 days. At the end of the study waa counted CFU-F (≥ 50 cells in the colony), using an inverting microscope and was conducted morphological analysis of colonies [46].

Morphological Examination of Preparations of Colony Forming Units

Cytological preparations of CFU-U are subjected to morphological analysis using the coloring in May-Grunwald [46]. Cytological preparations of CFU-GEMM, CFU-G painted on Wright studied peroxidase (myeloid line) and non-specific esterase (monocyte-macrophage line) [46, 47]. Erythroid cells line drugs CFU-GEMM identified Wright stain and were subjected to staining benzidine dihydrochloride [47]. Preparations of CFU-F were stained with azure II-eosin [46].

STATISTICAL ANALYSIS

Data are expressed as mean ± standard error of mean. Statistical variations were determined by analysis of variance (ANOVA) and Student's *t*-test. Values of $P < 0.05$ were considered significant.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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