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Pericytes and Smooth Muscle Cells Circulating in the Blood as Markers of Impaired Angiogenesis during Combined Metabolic Impairments and Lung Emphysema

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The changes in endothelial progenitor cells and progenitor cells of angiogenesis, pericytes and smooth muscle cells, were studied in female C57BL/6 mice with a combination of metabolic impairments induced by injections of sodium glutamate and lung emphysema modeled by the administration of cigarette smoke extract. It was observed that sodium glutamate significantly enhances pathological changes in the lungs (inflammation and lung emphysema) induced by the administration of cigarette smoke extract. Recruiting of endothelial progenitor cells ($CD45^-CD31^+CD34^+$ and $CD31^+CD34^+CD146^-$) and progenitor cells of angiogenesis ($CD45^-CD117^+CD309^+$) was registered in the injured lungs. Angiogenesis impairment induced by combined exposure is related to altered migration of pericytes ($CD31^-CD34^-CD146^+$) and smooth muscle cells ($CD31^-CD34^+CD146^+$) in emphysema-like enlarged lung tissue.

Key Words: *metabolic impairments; lung emphysema; endothelial progenitor cells; pericytes; smooth muscle cells*

Metabolic syndrome (MetS) is a complicated clinical state with abdominal visceral obesity as one of the main mechanisms [10]. About 20-30% of adult population of the world suffers from obesity and this number is constantly increasing [5]. Several investigators indicate that obesity promotes an increase in the reactivity of the respiratory pathway and development of various respiratory pathologies, such as chronic obstructive pulmonary disease (COPD), asthma, and other lung diseases [8,9]. It is considered that 10% of patients with obesity have COPD [12]. Moreover, obesity is often registered in patients with COPD and promotes the development of respiratory symptoms [7].

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MetS and obesity are often followed by the development of vascular complications. A decrease in the number of endothelial cells circulating in the blood and impairment in their functioning are registered in the patients with MetS and obesity [3]. On another hand, the impairments in endothelial function of lung arteries is observed in patients with COPD [2].

The combination of MetS and COPD is often observed in clinical practice, and the number of these cases is constantly increasing [1]. However, little is known about common pathological mechanisms of these disorders. It is not clear yet how MetS factors contribute to COPD progression. The lack of this knowledge makes the development of effective approaches to treatment of patients with MetS and COPD more difficult.

Here we studied the changes in microvasculature and endothelial progenitor cells during successive modelling of metabolic impairments and lung emphysema.

MATERIALS AND METHODS

The experiments were performed on female C57BL/6 mice from the Breeding Center of Biomodelling Department, E. D. Goldberg Research Institute of Pharmacology and Regenerative Medicine (veterinary certificate was obtained). All the manipulations were conducted in accordance to the European Convention on the protection of vertebrate animals used for experiments or other scientific purposes. The investigation was approved by the Committee for the Control of Keeping and Usage of Laboratory Animals of the E. D. Goldberg Research Institute of Pharmacology and Regenerative Medicine (IACUC protocol No. 114062016). Animal birth was taken as day 0 of the experiment.

Metabolic impairments (MD) were induced by the subcutaneous administration of sodium glutamate (MSG; Sigma) in a dose of 2.2 mg/g from day 1 to 10 of life [4]. Control animals received the same volume of saline. Li index was calculated at day 124 [4]. The index calculated as a cube root from the body weight (g)×nasoanal distance (mm) (equal or lower than 0.3) corresponded to the normal level. Females with Li index of more than 0.3 were considered as obese and included to the experiment.

On day 126 of the experiment, lung emphysema was induced by the course administration of LPS (Sigma) and cigarette smoke extract (SCE). LPS in a dose of 3 μg/mouse in 50 μl of phosphate buffer and 50 μl SCE were administered intratracheally. LPS was administered on days 126 and 129 of the experiment. SCE was administered on days 127, 130, 133, 136, 139, 142, 149, 156, 163, and 170 of the experiment. SCE was obtained from L&M cigarettes in accordance to the previously described method [15].

The animals were randomized to 4 groups: group 1, mice receiving physiological saline (intact control, n=9); group 2, mice receiving LPS and SCE (SCE, n=10); 3, mice receiving sodium glutamate (MD, n=10); group 4, mice with MD receiving LPS and SCE (combined pathology, n=10). All animals were sacrificed on day 188 of life by CO_2 overdose.

The development of MD was controlled by the blood glucose level, glucose tolerance test, and lipid profile. The impairments in the histology of lung tissue, presence of edema, inflammatory infiltration, venous stasis, and enlargement of vascular and bronchial walls were evaluated in the morphological study. The following primary antibodies were used for the immunohistochemical study: polyclonal antibodies to a membrane protein CD31, polyclonal antibodies to α1-antitrpsine (A1AT), and monoclonal antibodies to pan-cytokeratin (AE1/AE3) (Abcam). The analysis of obtained images and count of cells expressing the studied antigens were performed using the ImageJ software.

Mononuclear cells were isolated from the blood, bone marrow, and lungs of mice as described previously [15]. The expression of the surface markers CD45, CD31, CD34, CD146, CD309, and CD117 was measured using a FACSCanto II flow cytofluorimeter and FACSDiva software (BD Biosciences).

The *in vitro* analysis of programmed cell death (apoptosis) of $CD31^+$ cells from murine lungs was performed using the Cytation 3 Cell Imaging multitask reader (BioTek Instruments, Inc.). For this purpose, endothelial cells of the lungs were isolated, magnetically sorted, and cultivated in accordance to the protocol [15]. After the incubation period, endothelial cells of the lungs were treated with fluorescence dyes Hoechst 33342, Annexin V-iFluor 350, 7-AAD, and CFSE. Then, fluorescence was visualized using Cytation 3 and objectives 4× and 20× with the following analysis of the cells with the Gen5 software for data analysis.

Statistical analysis of the data was performed by the standard methods of variational statistics.

RESULTS

The administration of sodium glutamate to newborn mice damages the arcuate nucleus of the hypothalamus and reduces the level of neuropeptide Y regulating the transmission of signals from leptin and insulin [11]. Severe obesity and diabetes mellitus accompanied by hyperglycemia and hyperinsulinemia develop in these animals [14]. The effects of sodium glutamate in rodents are similar to the clinical pattern of MetS in patients [13,14].

The administration of sodium glutamate was followed by an increase in atherogenic index, concentrations of triglycerides and LDL, and a decrease in HDL level in blood serum of female mice on day 124 of the experiment (Fig. 1, *a-c*). Glucose level in the blood rose under these conditions (Fig. 1, *d*). Thus, sodium glutamate induced dyslipidemia, obesity, and hyperglycemia in mice, which can be considered as MD.

In accordance to modern theory, COPD is a result of chronic inflammation and trauma of alveolar endothelium, which can develop also as a response to long-term exposure to nicotine and tar. These etiological factors of COPD were modelled by SCE administration. LPS was additionally administered to enhance lung inflammation. The histological study allowed to observe venous stasis and inflammatory infiltration (besides macrophages and lymphocytes, neutrophils were found in inflammatory infiltrate), and enlargement of vascular and bronchial walls in the lungs of mice with MD receiving LPS and SCE compared to

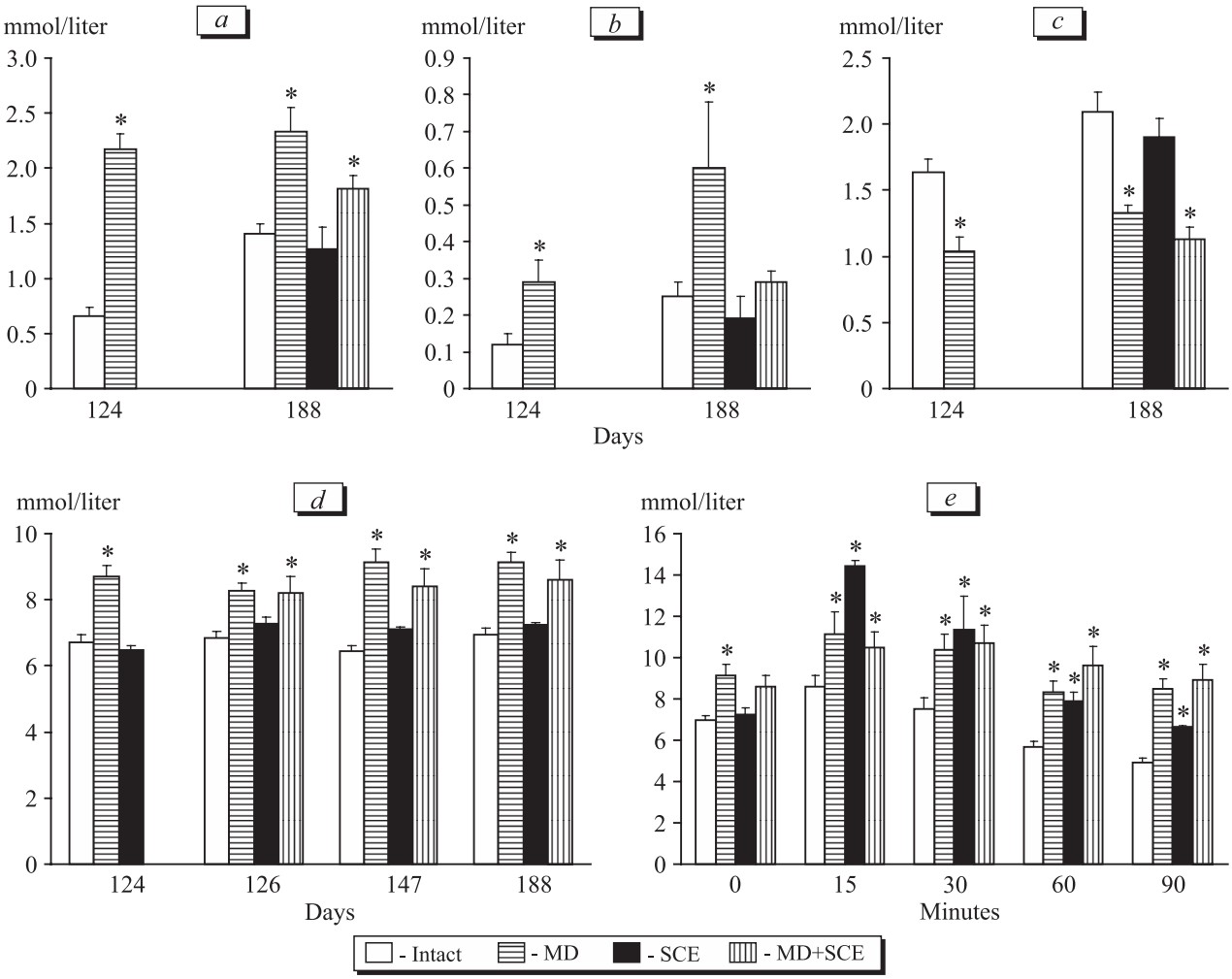

Fig. 1. Lipid profile in female C57BL/6 mice. *a*) Triglyceride level; *b*) LDL level; *c*) HDL level; *d*) glucose level in the blood during MD; *e*) glucose level in the blood during glucose tolerance test. *$p<0.05$ in comparison with intact control.

the intact control (Fig. 2, *a*). Moreover, emphysema was observed in the upper, medium, and lower parts of the lungs on day 188 of the experiment (Fig. 2, *b*). The intensity of impairments in the microvasculature, inflammation, and emphysema area in animals receiving LPS or SCE was lower than in mice with combined pathology. No pathological changes were observed in the lungs of mice receiving sodium glutamate.

Immunohistochemical study showed a decrease in the expression of markers of endothelial cells (CD31) and epithelial cells (pan-cytokeratin — AE1/AE3) in mice with the combined pathology. These data indicated the injury of alveolar epithelium and endothelium. It is known that A1AT prevents enzymatic damage of the epithelial cells. A significant decrease in A1AT expression in the alveolar tissue by 50% was observed in mice with MD receiving LPS and SCE compared to the intact control (Fig. 2, *c*).

Finally, programmed cell death (apoptosis) of $CD31^+$ cells from the lungs of intact mice and mice with the combined pathology was evaluated *in vitro* on day 188 of the experiment (Fig. 3, *a*, *b*). Modelling of the combined pathology significantly increased (by more than 3 times, $p<0.01$) the number of apoptotic $CD31^+$ endothelial cells compared to the intact control (Fig. 3). Esterase activity of $CD31^+$ cells reduced in mice with the combined pathology compared to the intact control (by 88%, $p<0.01$).

Therefore, the administration of sodium glutamate, LPS, and SCE was followed by the inflammation and emphysema in the lungs, and damages in the alveolar endothelium and epithelium. These changes were typical for mice receiving LPS and SCE. However, the intensity of inflammation and lungs emphysema in this group animals was significantly lower than in mice with the combined pathology. No pathological changes were observed in the lungs of mice with MD (Fig. 2).

Previous investigations [6] showed an increase in the number of endothelial progenitor cells (EPC;

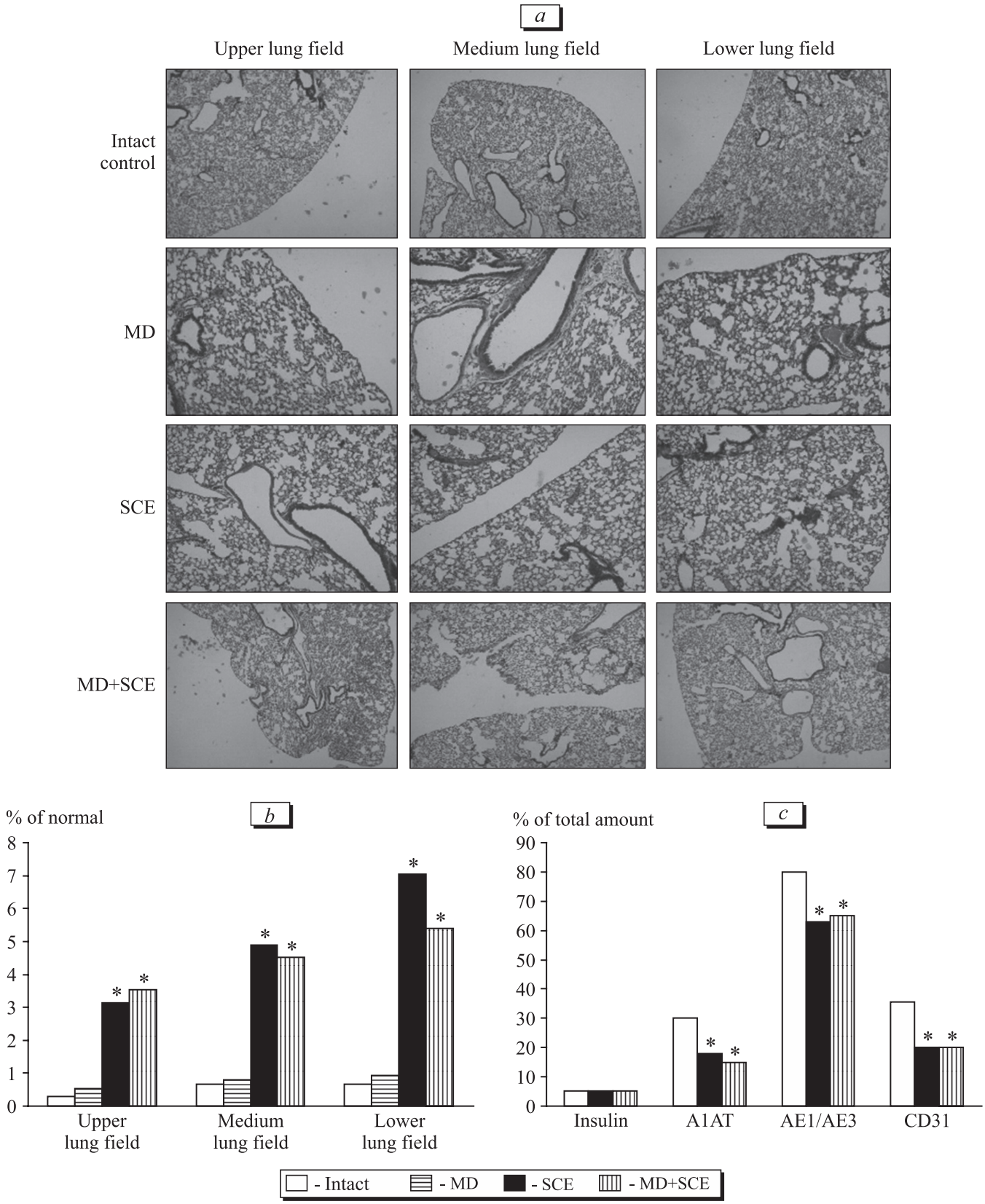

Fig. 2. Morphological pattern of the left lung (*a*), area of emphysema (*b*), and relative number of cells expressing specific antigens (*c*) in female C57BL/6 mice. Staining with hematoxylin and eosin, ×100. The samples were prepared on day 188 of the experiment. *$p<0.05$ in comparison with intact control.

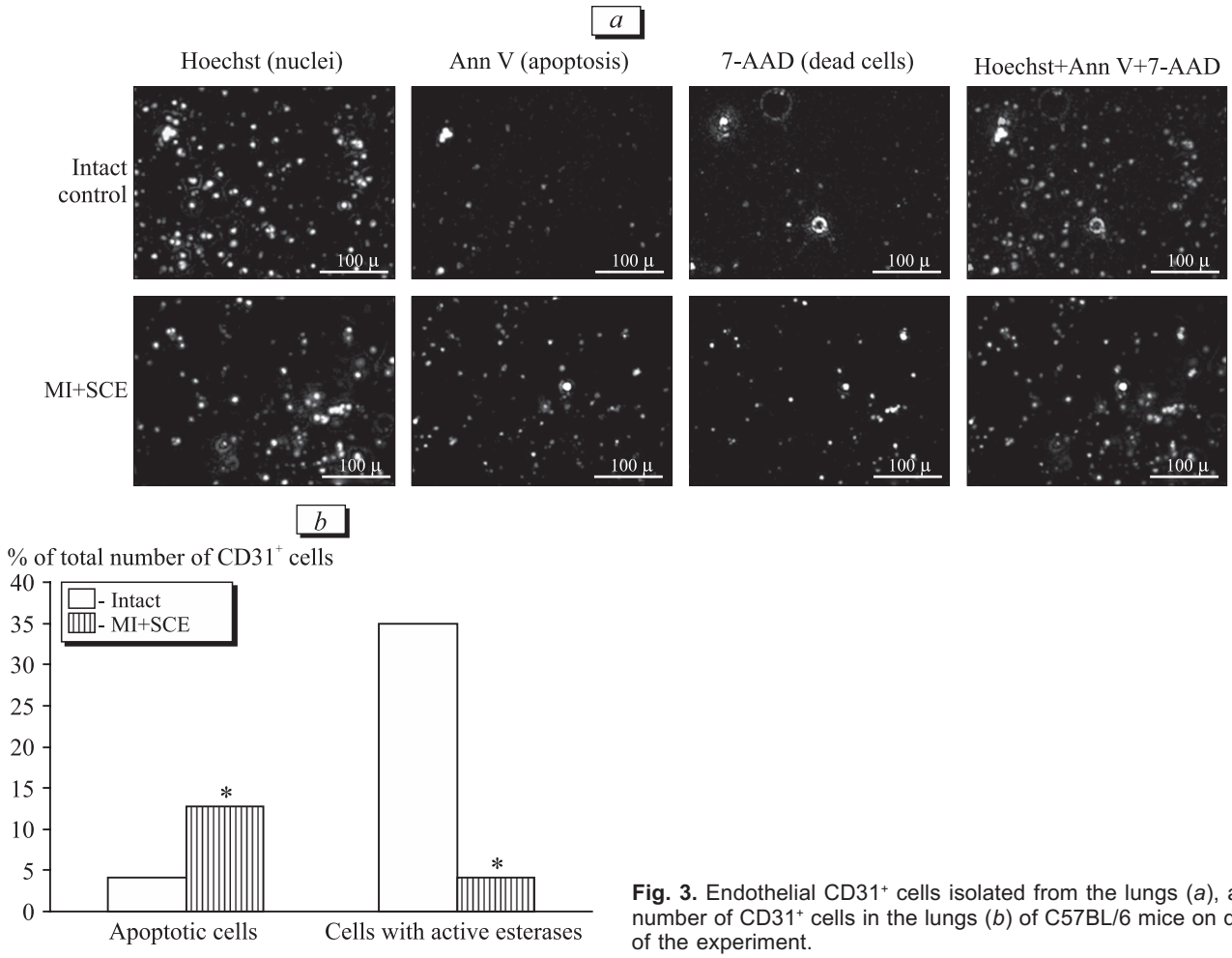

Fig. 3. Endothelial $CD31^+$ cells isolated from the lungs (*a*), and the number of $CD31^+$ cells in the lungs (*b*) of C57BL/6 mice on day 188 of the experiment.

$CD34^+CD309^+$, $CD34^+CD309^+CD133^+$) circulating in the blood of patients with COPD. Our early studies revealed a decrease in the number of $CD45^-CD31^-CD34^+$ EPC cells and progenitors of angiogenesis ($CD45^-CD309^+CD117^+$) in the blood of mice with SCE-induced lung emphysema. However, no increase in the number of immature endothelial cells was observed in the lung tissue [15]. These data indicate the impairment of regeneration of lung endothelium during COPD related to the changes in mobilization and migration of progenitors of endothelial cells to the damaged lungs.

In our study, COPD was associated with dyslipidemia, obesity, and altered glucose metabolism. It might be suggested that the impairments in regeneration of the alveolar tissue during the exposure to SCE differs from it during the combined pathology. Our hypothesis is in line with the data on the distribution of phenotypically different EPC in tissues. Subsequent modelling of MD and lung emphysema was followed by recruiting of EPC ($CD45^-CD31^+CD34^+$, $CD31^+CD34^+CD146^-$) and progenitor cells of angiogenesis ($CD45^-CD117^-CD309^+$) from the bone marrow to the injured lungs of mice at the day 188 of the experiment (Table 1). Previous studies [2] showed the direct connection between EPC recruiting and inflammatory cytokines. It cannot be excluded that the mobilization and following migration of EPC to murine lungs damaged by cigarette smoke directly correlates with the secretory ability of inflammatory cells. These cells might be also mobilized from the depot, such as fatty tissue. It is known that fatty tissue is involved in the processes of cell distribution during MD and obesity.

Despite the increase in the number of phenotypically different progenitors of endothelial cells in the lung tissue, the damages in lung endothelium in mice with the combined pathology remained. It might be related to the impairments in the differentiation of EPC and intracellular contacts playing the pivotal role in angiogenesis. Lately, it is considered that pericytes and vascular smooth muscle cells together with EPC contribute to the restoration of normal structure and functioning of damaged lungs. We observed a decrease in the number of pericytes ($CD31^-CD34^-CD146^+$) and smooth muscle cells ($CD31^-CD34^+CD146^+$) in the lungs of mice with the combined pathology. How-

TABLE 1. Number of Stem and Progenitor Cells (% of All Stained Mononuclear Cells) in the Blood, Bone Marrow, and Lung Tissue of Female C57BL/6 Mice with MD and Lung Emphysema on Day 188 of the Experiment (*M*±*m*)

Group	Endothelial cells		Progenitor cells of angiogenesis ($CD45^-CD117^+CD309^+$)	Pericytes ($CD31^-CD34^-CD146^+$)	Vascular smooth muscle cells ($CD31^-CD3^+CD146^+$)
	$CD45^-CD31^+CD34^+$	$CD31^+CD34^+CD146^+$			
Lung tissue					
Intact control	3.82±0.25	4.08±0.27	1.05±0.07	1.93±0.13	1.09±0.06
MI+lung emphysema	8.01±0.53*	6.15±0.41*	4.92±0.33*	0.89±0.06*	0.82±0.05
Bone marrow					
Intact control	0.27±0.02	12.96±0.86	0.47±0.03	7.11±0.47	5.34±0.36
MI+lung emphysema	0.69±0.05*	8.39±0.56*	0.40±0.03	5.92±0.39	4.90±0.33
Blood					
Intact control	0.93±0.06	22.79±1.52	—	2.57±0.17	0.74±0.05
MI+lung emphysema	0.10±0.01*	11.76±0.78*	—	29.66±1.98*	1.80±0.12*

Note. The results of three independent experimental series are presented. *$p<0.05$ in comparison with intact control.

ever, the number of the cells circulating in the blood significantly increased (Table 1). Insufficiency of regeneration mechanisms in the microvasculature during the combined pathology can be explained by the deficiency of pericytes and smooth muscle cells in the damaged lungs.

Therefore, hyperglycemia and dyslipidemia associated with combined MD and lung emphysema affect the cells contributing to angiogenesis. The recruiting of progenitors of endothelial cells in the alveolar tissue is accompanied by the impairment of migration of pericytes and smooth muscle cells. Thus, pericytes and smooth muscle cells can be markers of impaired regeneration of the alveolar endothelium in patients with combined MD and COPD. Some chemokines can restore the migration of pericytes and smooth muscle cells.

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