Creation of Experimental Models and Study of the Regeneratory Potential of Stem Cells on These Models

E. D. Gol'dberg, A. M. Dygai, V. V. Zhdanov, T. V. Vetoshkina, L. A. Gur'yantseva, T. Yu. Dubskaya, L. A. Ermolaeva, G. N. Zyuz'kov, E. V. Simanina, N. V. Sotnikova, L. A. Stavrova, E. V. Udut, T. I. Fomina, and T. Yu. Khrichkova

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Mobilization of mesenchymal stem cells and their more mature descendants, their differentiation into tissue-specific precursors, and the possibility of pharmacological stimulation of these processes were studied on models of some prevalent diseases. It was found that the development of any pathological process was associated with more or less pronounced activation of primitive and more differentiated mesenchymal precursors in the bone marrow, their mobilization into the peripheral blood, and increase in the content of regional precursors in organs exposed to pathogenic factors. Additional stimulation of stem cells with granulocytic colony-stimulating factor usually led to earlier or more pronounced increase in the content of mesenchymal progenitors in the bone marrow and peripheral blood. The content of tissue-specific and then fibroblast precursors in the regenerating organs also increased. This was paralleled by a decrease in the volume of developing connective tissue and degree of infiltration in these organs, while the percentage of tissue-specific structures increased.

Key Words: *mesenchymal stem cells; fibroblast precursors; myocardial infarction; chronic hepatitis; diabetes mellitus; encephalopathy; granulocytic colony-stimulating factor; regeneration*

Cell therapy is now at the early stage of development, but it is clear that its methods will be used in the treatment of many cardiovascular, gastrointestinal, nervous, endocrine, and locomotor diseases [3,5,10,14,19,20].

Introduction of polypotent progenitor cells into the body is, no doubt, fraught with unfavorable effects, including differentiation of these cells in an undesirable direction, immune conflict, and tumor development. This is particularly true for elements of fetal origin, but the probability of tumor transformation increases also after long-term *in vitro* culturing of postnatal stem cells, *e. g.* multipotent mesenchymal stromal cells [12]. In addition, culturing of these cells for induction of irreversible differentiation in a desired direction can reduce their proliferative potential and hence, the therapeutic efficiency [1,8].

A sufficiently large population of cells capable of developing into elements of different organs and tissues is present in an adult organism. Of all studied cells, mesenchymal stem cells (MSC; Fig. 1), no doubt, possess the greatest range of possible differentiation directions. Due to high plasticity of bone marrow (BM) MSC, they can transform, apart

Institute of Pharmacology, Tomsk Research Center, Siberian Division of Russian Academy of Medical Sciences

from connective tissue cells, into muscle cells and nervous tissue elements [10,16].

Regional precursors present in various organs during the postnatal period and providing the normal and reparative regeneration processes also have high proliferative and differentiation potential [6]. After alteration they migrate to the damaged zone, divide, maturate, and form cells of the corresponding tissue.

The ability to leave the tissue niche, circulate in the bloodflow and, getting into certain environment, to differentiate in the appropriate direction is the most important functional characteristics of stem cells [1,10]. Methods of pharmacological mobilization of hemopoietic precursors (stimulation of their release into the blood from depot) are described [15,17].

We studied the regularities of mobilization and differentiation of endogenous MSC and their more mature descendants into tissue-specific precursors and evaluated the possibility of pharmacological stimulation of these processes on models of some prevalent diseases.

MATERIALS AND METHODS

Experiments were carried out on Wistar rats and CBA/CaLac mice. Myocardial infarction was induced by ligation of the left coronary artery at the level of the first quarter of the distance from the pulmonary cone to the apex of the heart. The animals with electrocardiographically confirmed signs of coronary injury were taken into further experiments.

Toxic hepatitis was reproduced by intragastric treatment with 50% CCl_4 in olive oil in a dose of 2 ml/kg twice weekly for 3 weeks. Liver injury was diagnosed by serum levels of AST, ALT, and alkaline phosphatase (AP).

Diabetes mellitus was induced by 4 daily subcutaneous injections of alloxan (300 mg/kg) and its development was controlled by the blood and urine glucose levels.

Encephalopathy was formed under conditions of severe hemic hypoxia (150 mg/kg phenylhydrazine hydrochloride intraperitoneally) and verified by the development of amnesia in passive avoidance performance and disorders in the orientation and exploratory behavior in the "open field" test. Granulocytic colony-stimulating factor (G-CSF, Vektor Company in collaboration with Institute of Pharmacology) was injected to experimental animals subcutaneously (100 μg/kg) daily starting from the next day after the end of simulation of each disease.

The morphology and function of damaged organs was evaluated in various periods after disease modeling.

The content of MSC in the BM and peripheral blood was evaluated by the method of limiting dilutions. To this end, cell material (peripheral blood myelokaryocytes and mononuclears) in different concentrations was inoculated into plates and cultured for 6 weeks, the medium being replaced twice weekly. After incubation fibroblast-like cells were counted in each well. If their count was more than 10, the well was considered "positive", if less than 10, as "negative" [13]. The incidence of MSC in the BM and peripheral blood was evaluated using universal linear model for Poisson's distribution [11]. The content of committed fibroblast precursors was studied by the standard colony-forming method *in vitro* [2]. The content of regional precursors in the myocardium, liver, pancreas, and brain was evaluated by the clonal methods.

The data were statistically processed by methods of variation statistics using Student's *t* test. If the distribution of variants differed from the normal, nonparametric Wilcoxon—Mann—Whitney's method was used for evaluating the significance of differences between the samples [4].

RESULTS

Pronounced electrocardiographic changes developed in rats 3 h after coronary artery ligation. The *T* wave amplitude sharply increased, indicating the development of significant ischemic lesions in the myocardium. This was paralleled by reduction of the *R* wave amplitude and appearance of pathological *Q* wave (in 42% animals). In some cases, *QRS* complex was absent and *QT* complex appeared. These changes in the *QRS* complex reflected the formation of an extensive necrotic zone in the myocardium, while the electrocardiographic shifts together with pronounced morphological changes indicate the development of the acute stage of myocardial infarction.

The content of MSC in BM of animals with acute myocardial necrosis increased negligibly (Fig. 2). The number of their better differentiated descendants increased at later terms of the study. The content of fibroblast CFU in the peripheral blood virtually did not change. The content of clonogenic elements in myocardial tissue slightly increased throughout the experiment, but the detected changes were negligible. The formations consisting of at least 30 cells with fibroblast morphology were taken into consideration; these formations contained also few myocyte-like elements. Hence, activation of stromal progenitor cells in experimental myo-

cardial infarction did not lead to their mobilization into circulation and was insufficient for the increase in the content of regeneration-competent cells in the heart.

Three-week treatment with CCl_4 led to the development of stubborn destructive processes in the liver of experimental rats, which were paralleled by an increase in serum ALT activity on days 7, 14, and 21 and AST activity on days 7 and 14 after the end of treatment. Serum AP activity also increased.

Analysis of histological preparations of the liver on day 40 of the experiment showed small-droplet fatty degeneration, hepatocyte necrosis, development of cell infiltration of the liver parenchyma (mainly with macrophages and lymphocytes) of different degree, and fields of granulation tissue replacing dead cells. These changes indicated the development of chronic inflammatory process in the liver of experimental animals.

The count of committed fibroblast precursors in BM increased on days 7-10 after CCl_4 treatment (Fig. 3). The content of MSC in BM increased as early as on day 3. It seems that changes in various pools of stromal precursors are nonspecific and depend on activation of the stress-realizing systems during the development of liver disease induced by the toxic agent. In addition, the content of fibroblast precursor cells and the number of circulating MSC increased in the peripheral blood, this indicating mobilization of stem cells during this treatment. On the other hand, the content of regional precursor cells decreased in the liver, the decrease reaching the statistically significant level on day 10 of the experiment, after which the level of these precursor cells started to increase, reaching a statistically significant level only by the end of the experiment (day 14), when the toxic effects were obviously arrested.

Injections of alloxan to mice and rats led to elevation of blood glucose level as soon as on day 3 of observation period. This parameter was high virtually during the entire experiment, which was paralleled by the appearance of detectable levels of sugar in the urine.

Morphological study of the pancreas of these animals showed that alloxan treatment led to pyk-

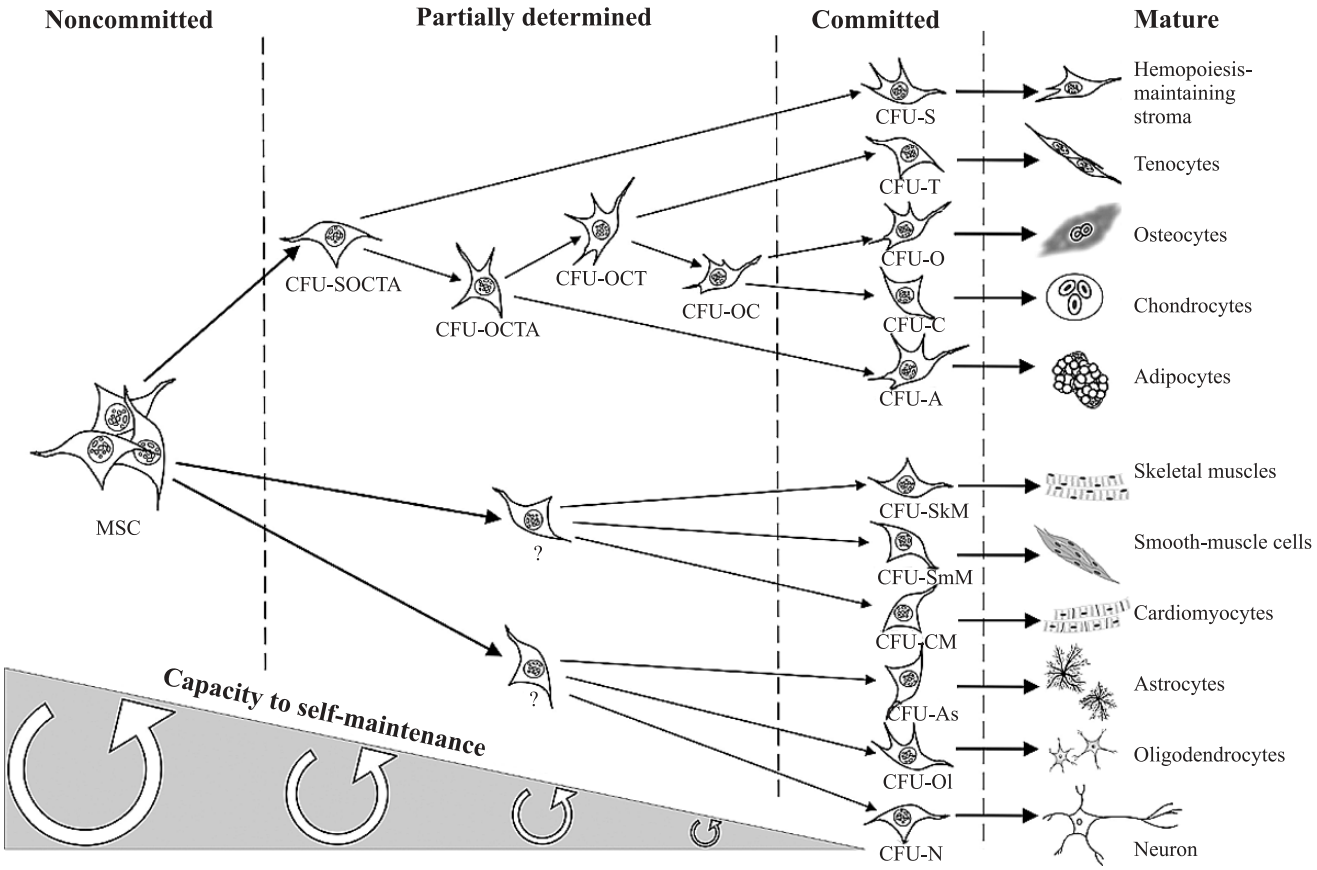

Fig. 1. Scheme of MSC differentiation [16]. CFU of: N: neurons; As: astrocytes; Ol: oligodendrocytes: SkM: skeletal muscles; SmM: smooth muscle cells; CM: cardiomyocytes; SOCTA: stromal cells, osteocytes, chondrocytes, tenocytes, adipocytes; OCT: osteocytes, chondrocytes, tenocytes; OC: osteocytes and chondrocytes; S: stromal cells maintaining hemopoiesis; O: osteocytes; C: chondrocytes; T: tendon cells; A: fatty cells.

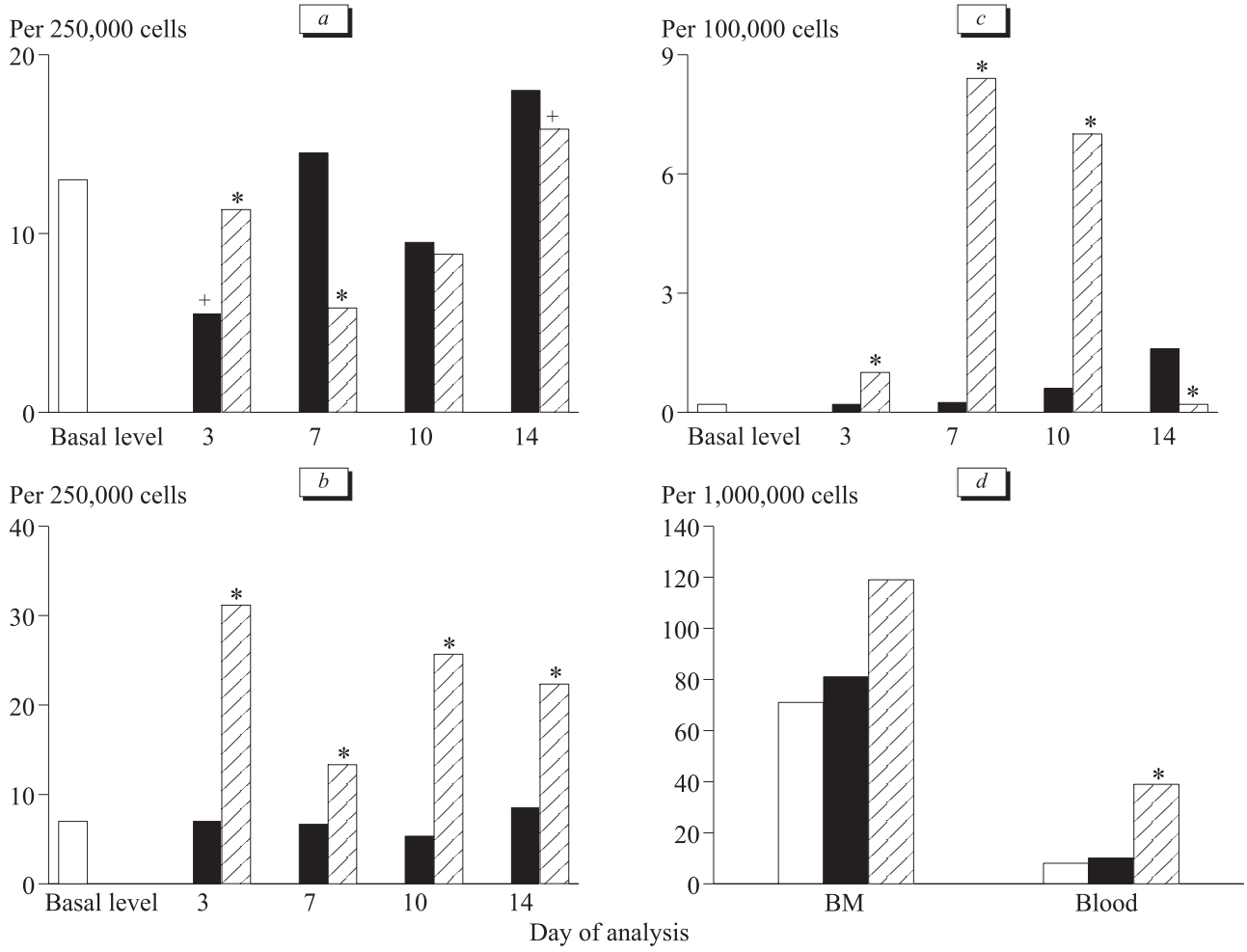

Fig. 2. Time course of fibroblast CFU in BM (*a*) and peripheral blood (*b*), clonogenic cells in the myocardium (*c*), MSC count on day 3 after treatment (*d*) in CBA/CaLac mice with acute myocardial necrosis. Light bars: basal level in intact animals; dark bars: infarction; cross-hatched bars: infarction+G-CSF. Here and in Figs. 3-5: $p<0.05$ *vs.* +intact, *infarction.

nosis of an appreciable part of cells in Langerhans islets. The percentage of pyknotic cells in the islets significantly surpassed the basal level and remained high until the end of observation. Moderate edema and hyperemia of the pancreatic tissue were observed at all terms of observation.

The number of fibroblast CFU in the BM increased from day 8 until day 15 after alloxan treatment, while the increase in the count of mesenchymal progenitor cells on day 8 was negligible (Fig. 4). On the other hand, the phenomenon of mobilization of committed and primitive stromal precursors into the blood was poorly expressed on this disease model.

The study of the pancreatic cell capacity to form colonies in culture showed an interesting fact: if the number of colony-forming parenchymatous units decreased over the course of observation (presumably, because of toxic effect of alloxan on these elements), the number of stromal precursors increased at later terms of the experiment (Fig. 4). Presumably, this phenomenon underlies the development of fibrous tissue in the pancreas during the proliferative phase of inflammation caused by alloxan.

Severe hemic hypoxia (injection of phenylhydrazine hydrochloride) was associated with the formation of encephalopathy, which was verified by the development of amnesia in passive avoidance performance and disorders in the orientation and exploratory behavior of animals in the "open field" test.

Encephalopathy led to a significant increase in the content of MSC in BM as early as on day 1 of the experiment, which however, did not significantly increase the content of these elements in the peripheral blood. Evaluation of the content of neural precursor cells in the paraventricular area of brain hemispheres in animals exposed to severe hypoxia showed an increase in their content, reaching a level of statistical significance on day 7 after treatment (Fig. 5).

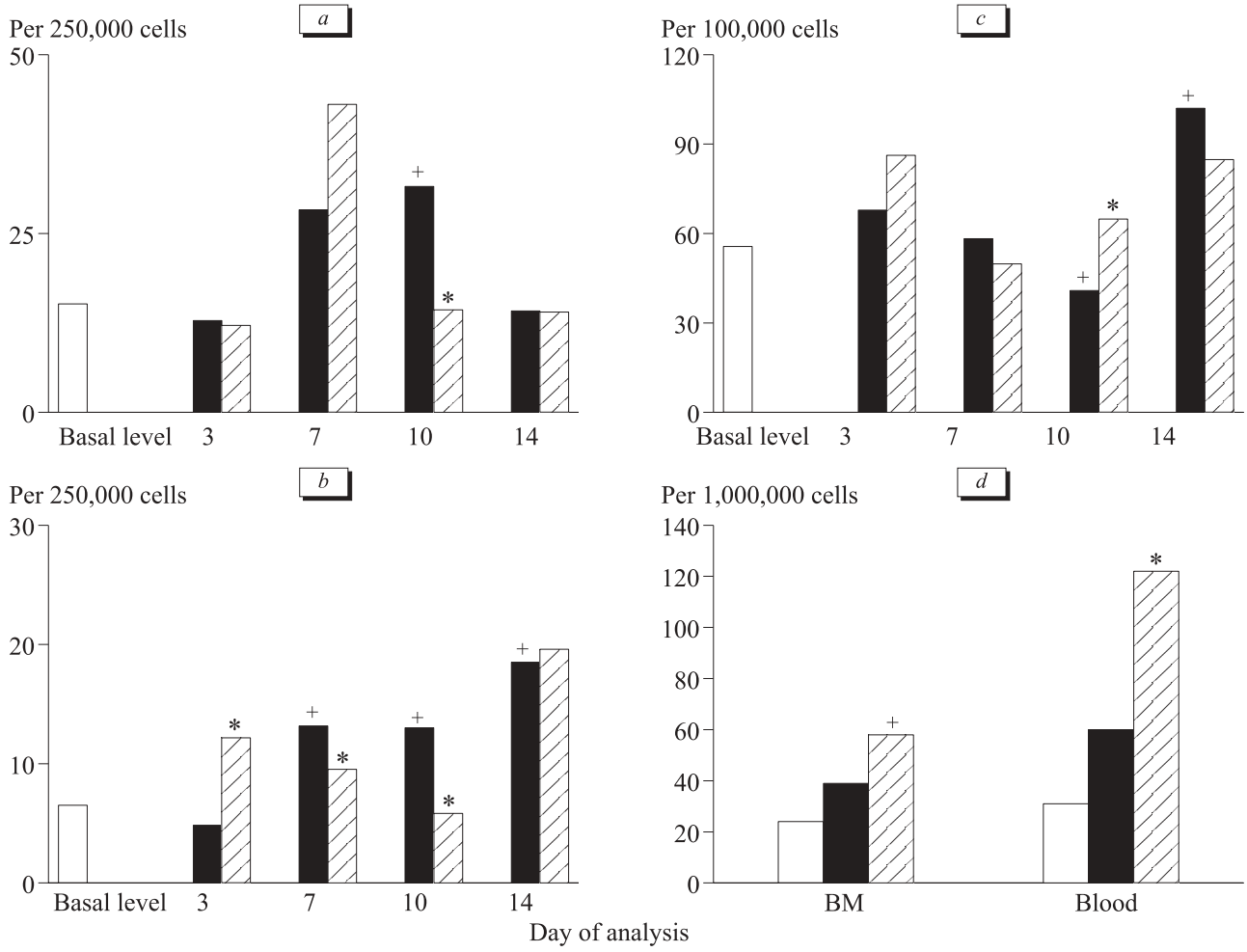

Fig. 3. Time course of fibroblast CFU numbers in BM (*a*) and peripheral blood (*b*), clonogenic cells in the liver (*c*), MSC count on day 3 after the end of CCl_4 treatment (*d*) in CBA/CaLac mice with chronic hepatitis.

Analysis of the data obtained on models of different pathological processes suggested that changes in the reserve systems of cell regeneration and mechanisms underlying these changes were nonspecific and universal, despite some characteristic features determined by the nature of the pathogenic factor. However, activation of BM stem cells, observed in the majority of cases, was insufficient for mobilization of MSC into the blood or for elimination of the suppressive effects of toxic agents towards target organs.

For additional stimulation of the regeneration-competent cells, the experimental animals were injected with G-CSF stimulating the release of hemopoietic precursors of different classes into the blood and increasing functional activity of progenitor cells of some other differentiation lines [15,18].

The use of recombinant cytokine in experimental myocardial infarction showed that G-CSF mobilized not only hemopoietic, but also stromal precursors (Fig. 2). The content of MSC in the peripheral blood increased significantly on day 3 after injection of G-CSF, indicating their release from depots. Parallel increase in the number of MSC in the BM suggests that stem cells released into the blood originate not only from the bone marrow, but also from other sources. The effect of G-CSF towards committed stromal precursors was similar and more pronounced. The content of regional precursors in myocardial tissue increased significantly on days 3, 7, and 10 of the experiment and decreased on day 14 after the start of G-CSF treatment. It seems that after mobilization of mesenchymal precursors under the effect of G-CSF the clonogenic cells accumulate in the focus of injury and differentiate into functionally competent myocardial cells.

The data obtained in the treatment of histological preparations confirmed this hypothesis. A connective tissue cicatrix formed 30 days after infarction at the site of necrotic zone in animals of both groups. In controls, the percentage of connective tissue elements in the total myocardial area on the preparations was 4.43%. In animals injected

with G-CSF, the postinfarction sclerosis was minor: collagen fibers in the cicatricial zone were alternating with cardiomyocytes and occupied just 0.39% of total area of the myocardium.

The course of G-CSF treatment in animals with toxic hepatitis was not associated with \significant increase in the content of MSC in the BM (Fig. 3). However, hemopoietin, similarly as CFU-F, increased their content in the peripheral blood on day 3 after the end of hepatitis modeling. The detected changes in the stem cell pool in the BM and peripheral blood, indicating mobilization of MSC, were paralleled by their homing into hepatic tissue, manifesting in a pronounced increase in the content of precursor cells in the liver on day 10 in comparison with animals injected with the solvent.

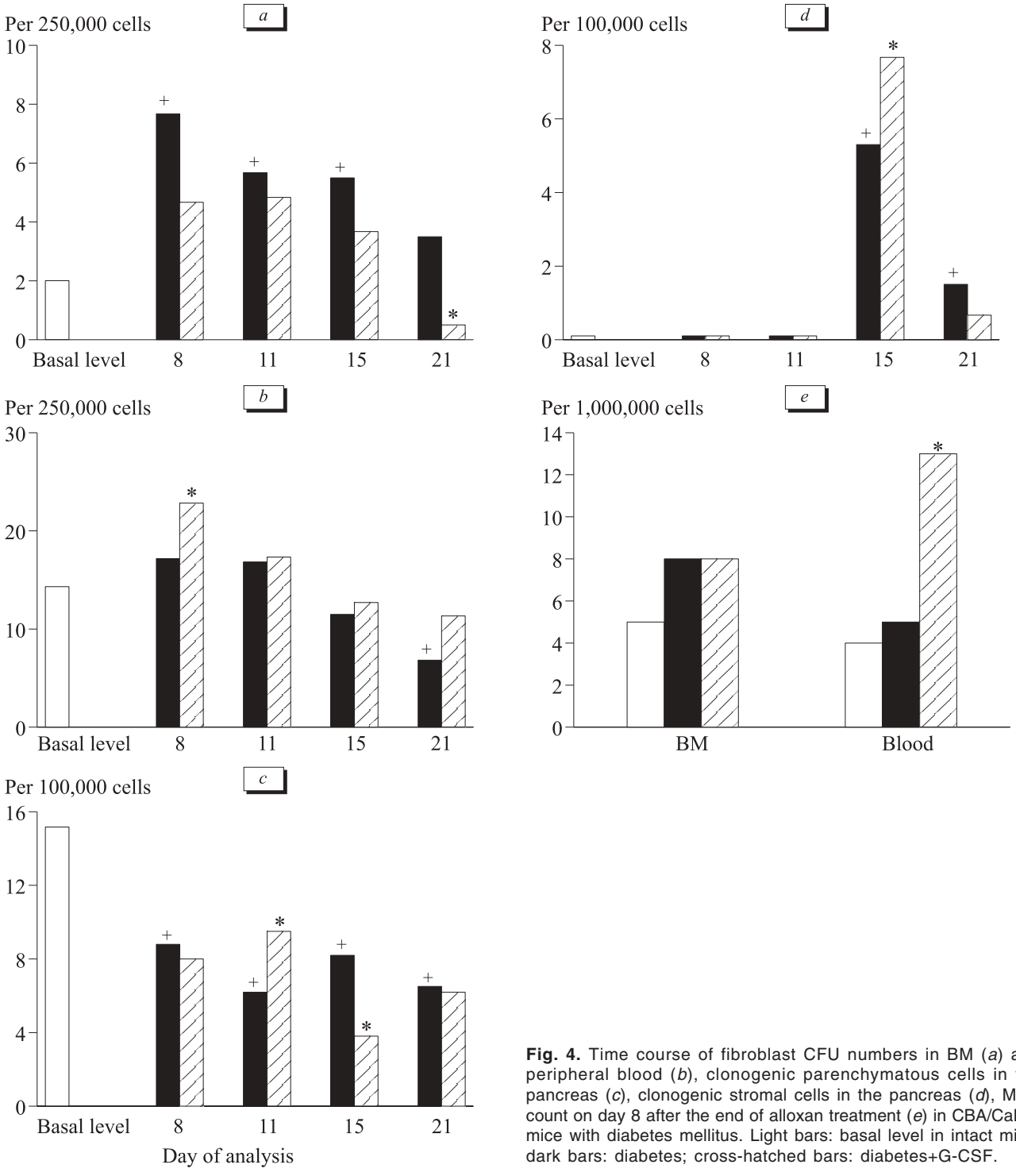

Fig. 4. Time course of fibroblast CFU numbers in BM (*a*) and peripheral blood (*b*), clonogenic parenchymatous cells in the pancreas (*c*), clonogenic stromal cells in the pancreas (*d*), MSC count on day 8 after the end of alloxan treatment (*e*) in CBA/CaLac mice with diabetes mellitus. Light bars: basal level in intact mice; dark bars: diabetes; cross-hatched bars: diabetes+G-CSF.

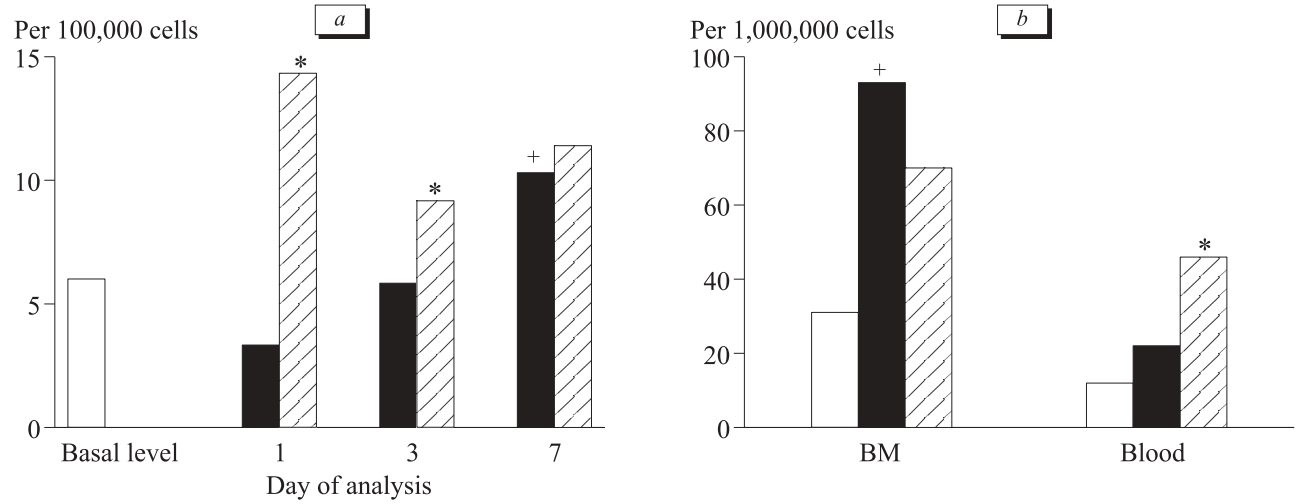

Fig. 5. Time course of neural precursor cells in the brain (*a*) and MSC count on day 1 after phenylhydrazine injection (*b*) in CBA/CaLac mice with hypoxic encephalopathy. Light bars: basal level in intact animals; dark bars: encephalopathy; cross-hatched bars: encephalopathy+G-CSF.

Morphological examination of the hepatic tissue showed that G/CSF treatment, virtually not modulating activity of inflammatory process, significantly reduced the degree of hepatic tissue sclerosis. Connective tissue occupied 1.41±0.16% of area after G-CSF treatment *vs.* 2.75±0.42% without treatment.

Hence, the hepatoprotective effects of G-CSF are presumably realized at the expense of stimulation, mobilization, migration, and determined homing of mesenchymal precursors into the liver. The precursors, in turn, can differentiate into hepatic precursors or create conditions for rapid development of resident hepatic clonogenic cells with their subsequent differentiation into mature hepatocytes [9,20].

The effects of G-CSF on the pool of primitive and better differentiated mesenchymal precursors were different in animals with alloxan diabetes (Fig. 4). The release of MSC into the blood under the effect of the cytokine did not lead to changes in their content in the BM, while mobilization of fibroblast CFU was associated with reduction of their number in the hemopoietic tissue.

Study of the content of regional precursors in the pancreas showed that G-CSF treatment first increased the number of units generating acinus-like (parenchymatous) formations in the culture and only then the number of stromal CFU. This fact seems to be serious evidence in favor of direct activation of the progenitor cell maturation into tissue-specific precursors by the growth factor without formation of additional microenvironmental elements from migrated precursors.

Study of histological preparations of the pancreatic tissue showed that treatment with G-CSF significantly decreased the number of pyknotic cells in the islets. In addition, the content of cells per unit of the islet area also decreased on day 21 of the experiment, which also indicated a decrease in pancreatic tissue infiltration.

Treatment of animals with severe oxidation insufficiency with G-CSF virtually did not modify the content of MSC in the BM on day 1 after the injection (Fig. 5). However, the preparation significantly increased MSC release into peripheral blood. Shifts in the MSC pool seemed to lead to their determined homing in the CNS. The content of neural precursors in the brain increased significantly on days 1-3 in phenylhydrazine-induced oxidation insufficiency. This phenomenon, in turn, was associated with disappearance of signs of psychoneurologic disorders (recovery of conditioned responses and orientation and exploratory behavior). On the other hand, G-CSF added to brain cell culture exhibited no direct effects on neural precursors *in vitro*.

These data indicate that a course of treatment with G-CSF, mobilizing MSC, promoted a more complete realization of their regeneratory potential, poorly utilized under conditions of simulated disease. After "stimulated" release from the depot, migration, and homing the mesenchymal progenitors differentiate into working tissue of respective organs, this preventing the development of connective tissue or manifestation of a chronic inflammatory process. The primitive progenitor cells are mobilized not only from the BM, but from other depots as well (for example, from adipose tissue [7]), while their more mature descendants are released into the blood mainly from the BM. In addition, presumably, migrated parental cells do not

differentiate into tissue-specific precursors by themselves, but form a specific microenvironment, thus creating conditions for their rapid development and subsequent differentiation into mature tissue elements [1,6].

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