Mechanisms of the Effects of Granulocytic CSF on Tissue Reparation during Chronic CCl_4-Induced Damage to the Liver

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Hepatoprotective effects of granulocytic CSF were studied using experimental model of CCl_4-induced hepatitis. It was found that treatment with granulocytic CSF increased the content of stromal precursors and mesenchymal stem cells in the bone marrow and peripheral blood with subsequent increase in the number of hepatic precursor cells in the liver. These findings attest to mobilization of progenitor mesenchymal cells and their migration into damages liver tissue, which accelerates its regeneration related to changes in the parenchyma, but not connective tissue development.

Key Words: *hepatitis; granulocyte colony-stimulating factor; stem cells*

Modern studies in the field of cell technologies assume possible development of a new treatment strategy, cell therapy, directed at restoration of functionally active tissue [4,7-9]. This strategy is very important for the treatment of chronic hepatitis, because of insufficiency of regeneration potential of the liver in this severe and protracted condition leading to the development of cirrhosis.

Mobilization of endogenous stem cells with their targeted homing into damaged tissues with the aid of pharmacological agents is a perspective trend of cell technologies. According to published data, granulocytic CSF (G-CSF) is a potent stimulators of mobilization of regeneration-competent cells among officially approved substances [6,10].

Here we studied hepatoprotective activity of G-CSF on the model of chronic CCl_4-induced hepatitis.

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MATERIALS AND METHODS

Experiments were carried out on 45 Wistar rats and 110 CBA/CaLac mice of both sexes aging 2 months. The mice (certified mouse strain) were obtained from the vivarium of Institute of Pharmacology (Tomsk Research Center). Hepatitis in rats was modeled by intragastric administration of 50% CCl_4 in olive oil (2 ml/kg) twice a week for 3 weeks (6 times). In mice, 20% solution was administered in a volume of 0.2 ml per mouse according to the same scheme. Animals of the background group received olive oil in equivalent volumes.

G-CSF (Vector Company in cooperation with Institute of Pharmacology, Tomsk Research Center) was injected subcutaneously in doses of 100 μg/kg in 0.5 ml saline for rats and 125 μg/kg in 0.2 ml saline for mice once a day for 5 consecutive days starting from day 19 of the experiment (the next day after the last administration of CCl_4). Controls received physiological saline according to the same scheme.

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In experiment on rats we evaluated animal mortality and weight gain on day 21 and 40 after the start of CCl_4 treatment. Activities of AST, ALT and alkaline phosphatase (AP) in blood serum were measured on days 7, 14, 21, 28, and 40. Morphological examination of the liver with calculation of the weight coefficient, *i.e.* ratio of liver weight (mg) to animal weight (g), was performed on day 40. During morphological examination, the relative infiltration area and relative area of the connective tissue in the liver parenchyma were determined [1].

Binding of mesenchymal precursors by cell component of the bone marrow microenvironment [3], the content of fibroblast CFU and mesenchymal stem cells in the bone marrow and peripheral blood, and the number of hepatic precursor cells in the liver of experimental and control animals were evaluated on days 3, 7, 10, and 14 after the last CCl_4 treatment.

The content of fibroblast CFU in the bone marrow tissue and peripheral blood was determined by culturing in semisolid methylcellulose medium supplemented with L-glutamine. It is now accepted that fibroblast CFU include not only stromal precursor cells, but also mesenchymal stem cells [9]. The cells were cultured for 7 days in a CO_2-incubator and the number of colonies (cell aggregations containing at least 50 spindle of stellate cells) was determined under an inverted microscope.

The content of mesenchymal stem cells in the bone marrow (at all terms of the study) and peripheral blood (on day 3 after the last CCl_4 treatment) was determined using the method of limiting dilutions [5] in our modification. To this end, the cells (myelokaryocytes and mononuclear cells of the peripheral blood) in different dilutions (maximum and minimum concentrations were 750,000 and 2500 cells per ml) were placed into plastic plates and cultured for 6 weeks in a CO_2-incubator; the medium containing L-glutamine, heparin, and fibroblast growth factor (FGF-basic) was replaced twice a week. After incubation the number of fibroblast-like cells in each well was determined. The wells containing more or less than 10 cells were considered as positive and negative, respectively.

The content of hepatic precursor cells was determined by cloning liver tissue in a medium containing L-glutamine, heparin, porcine multicomponent insulin, and stem cell factor (SCF). The colonies (round or irregular formation containing more than 30 cells) were counted after 10-day culturing in a CO_2-incubator.

The data were processed by methods of variation statistics using Student's *t* test and nonparametric Wilcoxon—Mann—Whitney *U* test. The incidence of mesenchymal stem cells in the bone marrow and peripheral blood was determined using a linear model for Poisson distribution [5,8].

RESULTS

Rat death in both groups was noted starting from the 3rd administration of CCl_4; to the end of the experiment 21.2 and 15.1% rats died in the control and experimental group, respectively. Pathomorphological study of the liver from dead animals revealed macroscopic signs of liver degeneration: enlargement and abnormal color of the organ, kaolin-like consistency, and infiltration foci. Weight gain on days 21, 28, and 40 was higher in animals receiving G-CSF compared to the control group, although this parameter was lower than in healthy animals of the background group.

Biochemical tests of blood serum in both groups revealed increased ALT activity on days 7, 14, and 21 and increased AST activity on days 7 and 14 of the experiment. Damage to the liver in both groups determined high AP content in the serum on days 7, 14, and 21 of the experiment. Treatment with G-CSF starting from day 19 of the experiment decreased ALT and AST activities relative to the control level only on day 40, and AP activity on days 28 and 40.

Analysis of histological preparations of the liver in both groups on day 40 of the experiment revealed fine-droplet fatty degeneration, necrosis of hepatocytes, more or less pronounced cell infiltration of the liver parenchyma (primarily with macrophages and lymphocytes), and fields of granulation tissue replacing dead cell elements. In the control group the infiltration and connective tissue areas were 15.40±1.20 and 2.75±0.42%, respectively. Treatment with G-CSF had little effect on activity of inflammatory processes, but considerably decreased the degree of sclerosis in the liver tissue (Fig. 1). In animals receiving G-CSF the connective tissue occupied 1.41±0.16% of the total area.

The data of biochemical and histological tests attest to pronounced hepatoprotective activity of G-CSF preparation.

The content of fibroblast CFU in the bone marrow of control animals increased on days 7 and 10 of the experiment. Not only stromal precursors, but also true stem cells were present among these cells, which can be indirectly confirmed by increased number of mesenchymal stem cells in the hemopoietic tissue at the same terms (to 279.1 and 291.7% compared to healthy animals on days 7 and 10, respectively). These changes in the pool of stem cells are probably unspecific and are related to ac-

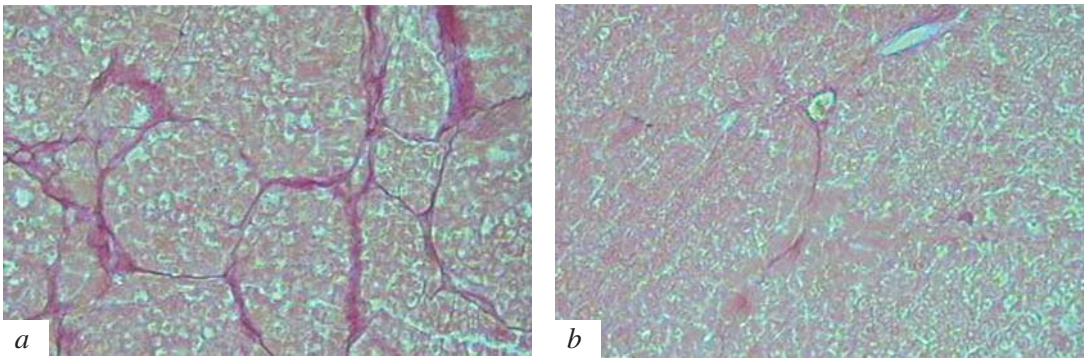

Fig. 1. Rat liver on day 40 after 6-fold CCl_4 treatment and injection of physiological saline (portal sclerosis; *a*) and granulocytic CSF (portal sclerosis is not pronounced; *b*). Picrofuchsin staining, ×200.

tivation of the stress-realizing system during the formation of liver pathology under the effect of the toxicant. Moreover, the content of fibroblast precursor cells in the peripheral blood increased on days 7, 10, and 14 of the experiment and the number of circulating mesenchymal stem cells increased on day 3, which attested to mobilization of stem cells of different maturity under these experimental

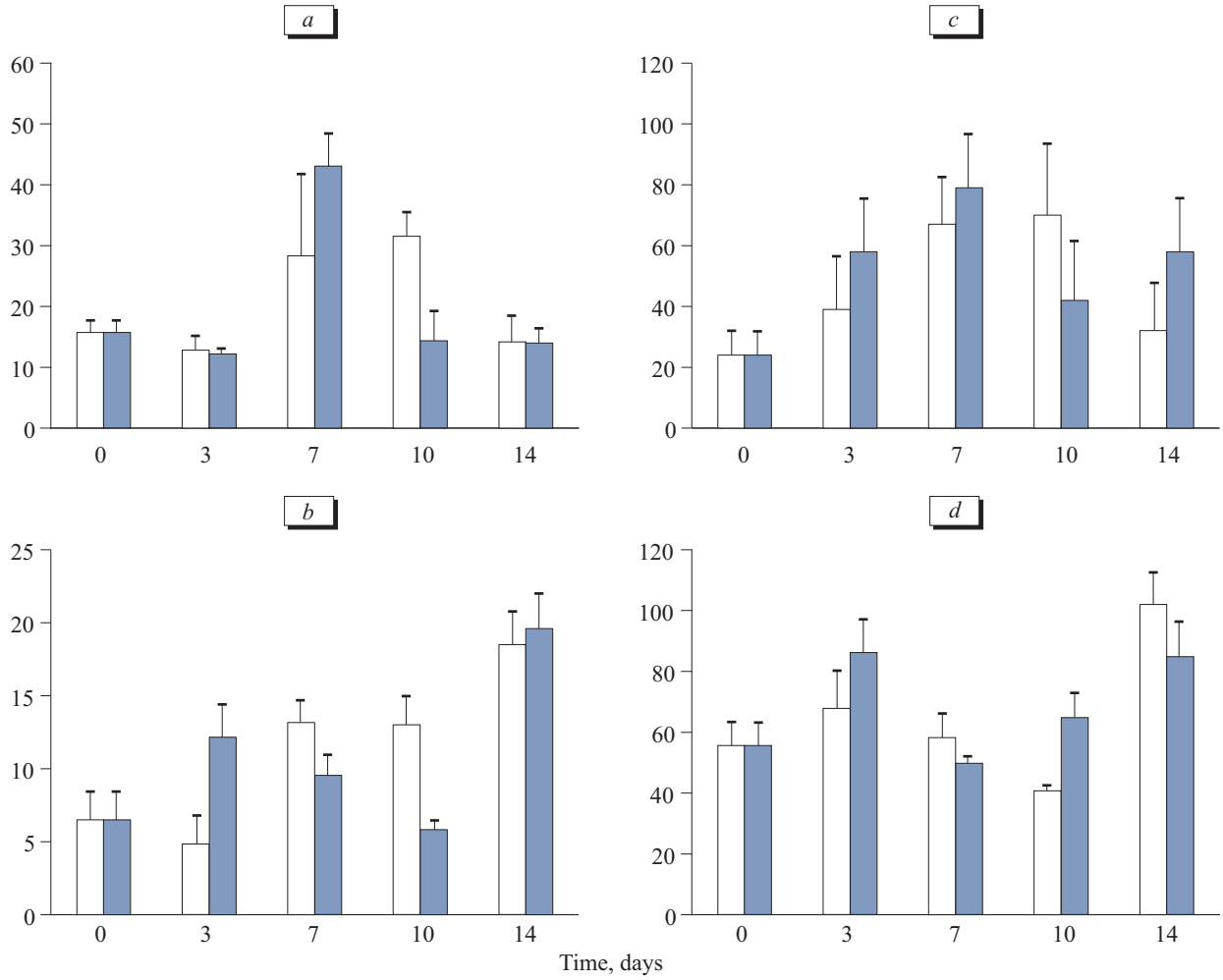

Fig. 2. Dynamics of the content of fibroblast CFU (per 250,000 myelokaryocytes) in the bone marrow (*a*), fibroblast CFU (per 250,000 mononuclear cells) in peripheral blood (*b*), mesenchymal stem cells (per 10^6 myelokaryocytes) in the bone marrow (*c*), and number of liver CFU (per 10^5 nuclears) in the liver (*d*) of CBA mice against the background of toxic hepatitis. Open bars: control; dark bars: experiment. Confidence intervals at p=0.05

conditions representing an extreme exposure. The release of these cells into circulation was observed even against the background of increased stem cell-binding capacity of the bone marrow microenvironment elements, which suggests the involvement of other mechanisms of mesenchymal precursor mobilization under conditions of CCl_4 intoxication. Evaluation of the content of regional precursors in the liver revealed a tendency to a decrease in the number of liver CFU; on day 10 this decrease became significant, while significant increase in this parameter was observed only to the end of the experiment (day 14), when toxic effect of CCl_4 disappeared. These data attest to activation of mechanisms of compensation of deep reserves (bone marrow stem cells) in chronic CCl_4 intoxication, which are probably insufficient and/or incompetent for complete removal of the toxic effects of CCl_4 on the liver tissue.

Short-term treatment with G-CSF significantly increased the content of mesenchymal stem cells in the bone marrow on day 7 of the experiment against the background of increased content of fibroblast CFU (day 7, Fig. 2). Hemopoietin increased only the content of fibroblast CFU in the peripheral blood on day 3 after the end of hepatitis modeling. These CFU contained also unipotent stromal precursors and true mesenchymal stem cells. The content of mesenchymal stem cells in the peripheral blood increased to a greater extent (to 403.2% of the level observed in healthy animals) than in animals receiving physiological saline. These shifts did not depend on the binding capacity of stromal elements of the bone marrow and its structural and functional organization. At the same time, the shifts in the pool of stem cells in the bone marrow and peripheral blood caused by the test preparation and attesting to mobilization and migration of mesenchymal stem cells were accompanied by their homing into the liver tissue. This manifested in increased content of precursor cells in the liver on day 10 to 158.8% relative to animals receiving the solvent.

Thus, our experiments demonstrated hepatoprotective properties of G-CSF. These properties are realized via stimulation, mobilization, migration, and determined homing into the liver of mesenchymal precursors capable of differentiation into hepatic precursors or creating conditions for accelerated development of resident liver CFU followed by their differentiation into mature hepatocytes.

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