Hepatoprotective Effects of Immobilized Granulocyte Colony-Stimulating Factor and Hyaluronidase Preparation and Their Mechanisms

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High hepatoprotective activity of granulocytic CSF and hyaluronidase immobilized using electron-beam immobilization technology was demonstrated on the model of CCl_4-induced hepatitis: the preparations produced anticholestatic, anti-inflammatory, and antisclerotic effects. These effects developed against the background of stimulation of bone marrow multipotent precursor cells and their mobilization into circulation accompanied by an increase in the content of parenchymatous progenitor cells in the liver. The most pronounced positive effect was observed in combined treatment with the test preparations.

Key Words: *chronic hepatitis; granulocyte colony-stimulating factor; hyaluronidase; nanotechnologies; progenitor cells; regenerative medicine*

Chronic hepatitis is one of the most prevalent, severe, and long-lasting liver diseases; regeneration reserve capacity of the liver tissue in this condition is often insufficient, which leads to replacement of functionally active parenchyma with the connective tissue and disturbances in structural organization of the organ and eventuates in cirrhosis of the liver [6]. Hepatoprotectors routinely used in clinical practice are often ineffective; however, a new approach, pharmacological stimulation of endogenous SC, was recently proposed [13]. Granulocytic CSF (G-CSF) is known to be the most promising modulator of SC functions [3]; it induces SC mobilization from tissue depots followed by their homing in the focus of injury and differentiation into specialized parenchymatous cells of the organ or tissue microenvironment regulating the state of the pool of regeneration-competent precursor cells [3,10]. We previously demonstrated the possibility of increasing SC reserve in the body and potentiation of hemostimulating properties of G-CSF and its mobilizing effects towards progenitor cells with hyaluronidase [2,7], the key enzyme determining cell behavior [7,12].

The use of hyaluronidase in clinical practice is limited due to its high immunogenicity and rapid inactivation by tissue inhibitors; G-CSG also produces a number of undesirable side effects. The newest method of immobilization of pharmacologically active substances on inert carriers (low-molecular-weight polymers) using a nanotechnology of electron-beam synthesis makes it possible to consider-

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ably reduce the risk of complications and increases bioavailability and efficiency of preparations. In light of this, we evaluated hepatoprotective effects and studied the mechanisms of action of immobilized hyaluronidase (iHD) and G-CSF (iG-CSF) and their combination on the model of chronic hepatitis induced by long-term CCl_4 treatment.

MATERIALS AND METHODS

Experiments were carried out on 2-month-old CBA/CaLac male mice (n=130) and male Wistar rats (n=49). Chronic hepatitis was induced by intragastric administration of 50% CCl_4 solution in olive oil in a dose of 0.2 ml per mouse and 20% solution in a volume of 2.5 ml per rat (5 doses, every 3 days over 15 days). Starting from day 16, experimental animals intragastrically received: 1) 100 μg/kg G-CSF for 10 days; 2) 50 U/kg iHD for 2 days; 3) 100 μg/kg G-CSF for 10 days and 50 U/kg iHD for 2 days. The preparations synthesized by Scientific Future Management Company (Novosibirsk) and Institute of Pharmacology (Siberian Division of the Russian Academy of Medical Sciences, Tomsk) were used. Immobilization of the molecules on a low-molecular (1.5 kDa) carrier was carried out using the nanotechnology of electron-beam synthesis employing a directed flow of accelerated electrons [5]. Control mouse and rat groups comprised 10 animals (administration of physiological saline) and 10 mice and 9 rats remained intact.

In experiments on rats we evaluated animal death and body weight gain; on days 21 and 40, serum activities of AST, ALT (with calculation of de Ritis coefficient AST/ALT), and alkaline phosphatase (AP) were measured routinely on a Cormay biochemical analyzer using Cormay and Vektor Best kits.

On days 21 and 40 after the start of CCl_4 poisoning, morphological study of the liver was performed with calculation of the weight coefficient (ratio of organ weight (mg) to liver weight (g) as an integral marker of hepatotropic effects of xenobiotics [8]. The liver was weighed and liver fragments were fixed in 10% neutral formalin and embedded in paraffin. Deparaffinized sections were stained with hematoxylin and eosin and with picrofuchsin (for connective tissue visualization) [8]. Using computer image analysis system we determined the number of infiltration cells on histological sections of the liver stained with hematoxylin and eosin and the area of the connective tissue on sections stained with pikrofuchsin. To this end, the number of infiltrate cells or area of pikrofuchsin-stained structures were determined on a standard section area (successive microphotographs of 10 fields of view taken using Digital microvideo camera input into a computer using Elekard software) [1].

In experiments on mice, the content of fibroblast CFU (CFU-F) in BM and peripheral blood and the content of liver cell precursors in the liver were evaluated on days 21, 23, and 26 of the experiment [4,11].

The data were processed by methods of variation statistics using Student's t test and nonparametric Mann–Whitney U test.

RESULTS

Rat mortality was observed starting from the 3rd administration of CCl_4 and by the end of the experiment it attained 10%. Autopsy of dead animals revealed enlarged yellowish liver of clay consistence with hemorrhagic foci and macroscopic signs of acute degeneration.

In rats receiving 5 doses of CCl_4 (by day 21 of the experiment), the weight coefficient of the liver, the integral marker of animal state, was considerably increased. This attests to the development of a sustained chronic liver pathology. The test preparations produced no positive effect on this parameter throughout the experiment; however, a tendency to less pronounced decrease in body weight was observed in treated animals in comparison with the control.

Histological analysis of the liver confirmed the development of toxic hepatitis in rats of the control group on day 21 of the experiment. Disordered lobular structure of the liver and monocellular hepatocyte necrosis were observed. In preserved hepatocytes, small- and large-droplet fatty degeneration was seen. Individual cells fussed and formed fatty cysts. Regenerative hypertrophy of hepatocytes was clearly seen. Infiltration (primarily with lymphocytes and macrophages) was diffuse.

On day 40 of the experiment, morphological signs of hepatitis in control rats were less pronounced than on day 21. Fatty degeneration of hepatocytes persisted, but became small-droplet. Lymphocyte and macrophage infiltration also persisted. The number of infiltrate cells and the area of the connective tissue significantly surpassed the control values (Fig. 1).

In rats receiving the test preparations, morphological changes in the liver were less pronounced. Fatty degeneration of the liver tissue persisted, but was primarily small-droplet. The predominant effects of the test preparations in individual administration were different. iG-CSF practically arrested the growth of collagen fibers at all terms of observation, but did not prevent accumulation of the infiltrate in the liver tissue on day 21. iHD had no appreciable effect on the relative area of the connective tissue, but actively reduced infiltration at the initial terms of the experiment (Fig. 1). Combined treatment with the test preparations significantly reduced the area of the connective tissue

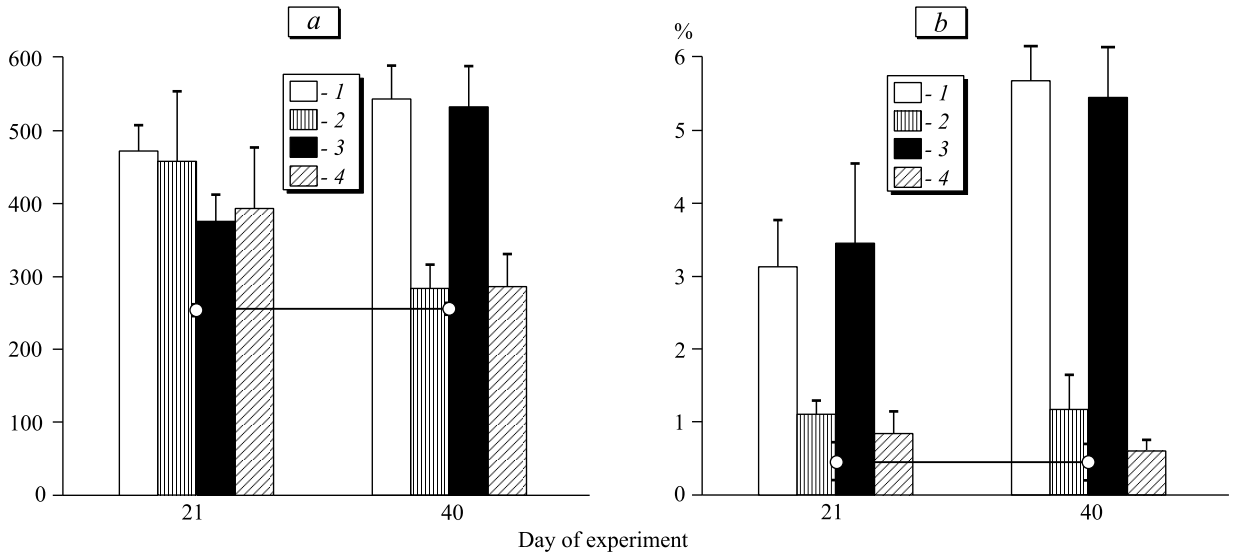

Fig. 1. Number of inflammatory infiltrate cells (*a*) and relative area of collagen fibers (*b*) in the liver of rats with chronic CCl_4-induced hepatitis on days 21 and 40 of the experiment. Here and in Figs. 2, 3: *1*) without treatment, *2*) iG-CSF; *3*) iHD; *4*) iG-CSF+iHD; horizontal line shows background value.

on histological sections in comparison with that in the control (practically to background values). The number of inflammatory infiltration cells tended to decrease in comparison with the control by day 21; then this parameter became significantly below the control and approached the background level (Fig. 1).

Increased AP activity in the serum and reduced de Ritis coefficient attested to severe metabolic disturbances in the liver of untreated rats on days 21 and 40 of the experiment. Treatment with the test preparations similarly reduced AP activity in comparison with the control group (Fig. 2), but had little effect on transaminase activities.

Thus, the most pronounced hepatoprotective effect was observed in case of combined treatment with iG-CSF and iHD. Apart from restored AP activity in the blood (anticholestatic effect), the preparations produced anti-inflammatory and antisclerotic effects.

Experiments on mice aimed at elucidation of the mechanisms of the hepatoprotective effects of immobilized preparations showed CCl_4 intoxication led to an increase in the count of fibroblast precursors in BM and significant decrease in the count of tissue-specific colony-forming cells in the liver on days 23 and 26 of the experiment, but produced no effect on the content of CFU-F in the peripheral blood of experimental animals (Fig. 3). Administration of iG-CSF induced accumulation of mesenchymal precursors in BM at all terms of the experiment, but did not change their content in the peripheral blood and liver tissue. At the same time, the preparation prevented the decrease in the content of liver CFU in cultures of liver cells. iHD considerably increased CFU content in all three cultures as soon as on day 21 of the experiment. By day 23, the content of precursors in the peripheral blood and liver returned to initial levels, while in BM it still considerably surpassed the background values. By day 26, the content of CSF-F in BM somewhat decreased, but still surpassed the control. In the peripheral blood, this parameter slightly increased, but this increase was less pronounced than on day 21. At the same time, the content of regional precursors in the liver tissue by day 26 attained 219% of the background level and 311% of the control. iHD induced enhanced growth of stromal elements in cultures of liver cells, which was not observed in other groups. The dynamics of the content of fibroblast precursors and liver CSF in the examined tissues after combined treatment with iG-CSF and iHD fully corresponded to that after iHD

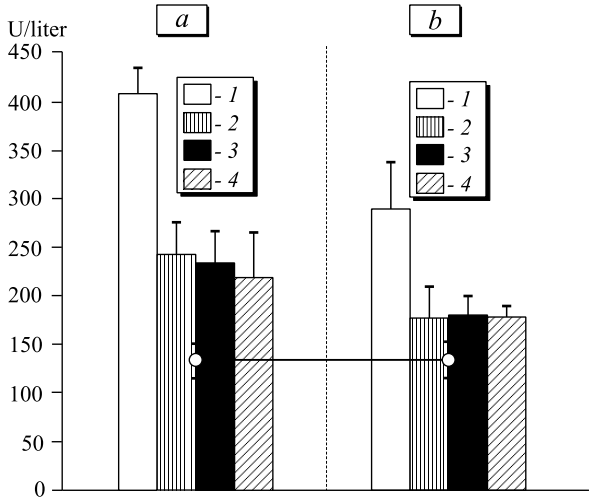

Fig. 2. Serum AP level in rats with chronic CCl_4-induced hepatitis on days 21 (*a*) and 40 (*b*) of the experiment.

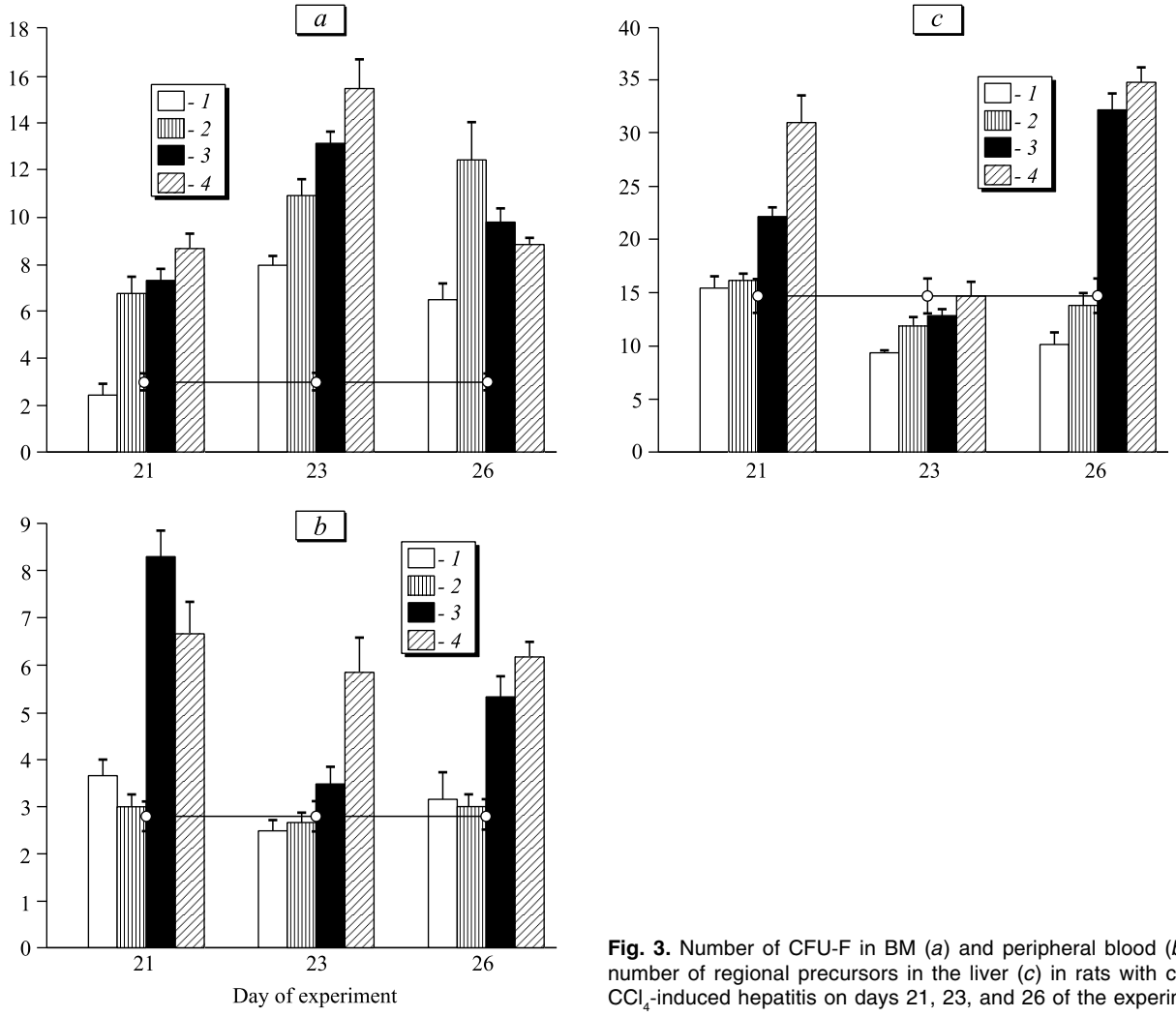

Fig. 3. Number of CFU-F in BM (*a*) and peripheral blood (*b*) and number of regional precursors in the liver (*c*) in rats with chronic CCl_4-induced hepatitis on days 21, 23, and 26 of the experiment.

treatment, but accumulation of precursors was more pronounced (Fig. 3).

Thus, both test preparations stimulated the cell renewal reserve systems involved in liver tissue recovery. The mechanisms of their effects are probably related to activation of BM mesenchymal precursor proliferation, their mobilization into peripheral blood, and targeted homing in the liver tissue, where they activate local regenerative mechanisms and prevent hepatocyte destruction. The most pronounced positive effect was observed in case of combined treatment with the test immobilized preparations. It should be noted that iHD preparation apart from its independent effect on precursor cells potentiated the effect of the cytokine.

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