# NANOTECHNOLOGY

# Mechanisms for Hepatoprotective Effects of Hyaluronidase Immobilized by the Nanotechnology Method of Electron-Beam Synthesis

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Immobilized hyaluronidase (nanotechnology method of electron-beam synthesis) exhibited high hepatoprotective activity on the model of $Cl_4$-induced hepatitis. This agent produced anticholestatic, anti-inflammatory, and antisclerotic effects. These effects were shown to accompany stimulation of multipotent bone marrow precursors, mobilization of these cells into the peripheral blood, and cell migration to the target organ increasing the number of parenchymal progenitor cells in the liver. The mechanisms for targeted migration of progenitor cells suggest a decrease in SDF-1 production by bone marrow stromal cells and increase in the synthesis of this factor by microenvironmental cells of the liver tissue.

**Key Words:** *chronic hepatitis; hyaluronidase; nanotechnologies; progenitor cells; regenerative medicine*

The worldwide incidence of liver diseases is very high. These disorders occupy the significant place in the morbidity and mortality structure. Low efficiency of hepatoprotective drugs necessitates the development of new pathogenetic methods and approaches to the therapy of liver diseases. A new approach to cell therapy of various diseases was developed by studying the properties and functions of multipotent precursor cells [2,7,14]. Pharmacological stimulation of endogenous stem cells is the most physiological method of regenerative medicine [8,9]. *In vitro* and *in vivo* observations showed that functions of various progenitor cells can be modified by hyaluronidase [4,6]. Under certain conditions, this enzyme cleaves hyaluronic acid of the intercellular matrix into polymers activating cell division and differentiation [13,15]. Moreover, hyaluronidase potentiates the inducing effect of exogenous factors on stem cell release into the blood.

Here we studied the hepatoprotective properties of hyaluronidase immobilized by the nanotechnology method of electron-beam synthesis (immobilized hyaluronidase, IMHD). We also evaluated the mechanisms for action of IMHD that are associated with functional activity of progenitor cells of various classes.

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## MATERIALS AND METHODS

Experiments were performed on outbred rats ($n$=56, body weight 250-300 g) and 2-month-old male CBA/CaLac mice ($n$=115, body weight 18-20 g). The animals were obtained from the nursery of the experimental biological clinic of laboratory animals (Institute of Pharmacology). Chronic hepatitis in rats was induced by intragastric administration of $CCl_4$ (2 ml/kg, 50% olive oil solution) twice a week for 3 weeks (6 injections). Liver injury in mice was induced by intragastric administration of $CCl_4$ (20% olive oil solution, 0.2 ml per mouse) under similar experimental conditions. IMHD was administered intragastrically on day 3 after the last treatment with $CCl_4$ (day 19 of the experiment). The rats received IMHD in a daily dose of 35 U/kg for the first 2 days; in the following 10 days, IMHD was given in doses of 35 and 50 U/kg at one-day intervals. The mice received IMHD in a daily dose of 50 U/kg for 2 days. IMHD was developed by the Scientific Features Management Company in collaboration with the Institute of Pharmacology. Enzyme molecules (Scientific Features Management Company) were immobilized on low-molecular-weight polyethylene oxide (1.5 kDa) by the nanotechnology method of radiational synthesis with a directed flow of accelerated electrons [8]. Control animals received an equivalent volume of distilled water (similarly to treatment with IMHD).

The mortality rate, body weight gain, and relative weight of the liver (liver weight/body weight ratio, mg/g) in rats were evaluated on days 21 and 40. The contents of AST, ALT, and alkaline phosphatase in blood serum were measured on days 7, 14, 21, 28, and 40. A morphological study of the liver was conducted on days 21 and 40. Computerized methods of graphic data processing were used to estimate the number of infiltrating cells in the standard area of liver sections after hematoxylin-eosin staining. The area of collagen fibers was evaluated on picrofuchsin-stained sections [1,10].

The number of mesenchymal precursors (fibroblast CFU) in the bone marrow and peripheral blood [5] and count of parenchymal stem cells in the liver (CFU-L) of mice [11] were estimated by the method of *in vitro* cloning on days 21, 23, and 26. Production of stromal cell-derived factor-1 (SDF-1) by tissue cells was evaluated from the content of this factor in conditioned media. EIA with R&D Systems kits was performed according to the manufacturer's recommendations.

The results were analyzed by methods of variation statistics (Student's $t$ test and Mann–Whitney $U$ test).

## RESULTS

Death of rats was observed after the 3rd treatment with $CCl_4$. By the end of observations, the mortality rate of these animals was 10%. Autopsy examination showed that the liver was enlarged, yellowich, and had clay consistency. We revealed the presence of focal hemorrhages and macroscopic signs of acute atrophy. Studying the general state of experimental rats showed that the development of toxic hepatitis is accompanied by a significant decrease in body weight gain. Body weight gain in IMHD-treated animals was much higher than

**TABLE 1.** Biochemical and Morphological Parameters of Outbred Rats with Experimental Liver Injury ($X \pm m$)

| Period, days | Group | Biochemical parameters | | | Morphological parameters | |
|---|---|---|---|---|---|---|
| | | ALT, μcat/liter | AST, μcat/liter | AP, U/liter | NCII, U | RACF, % |
| Baseline | | 0.30±0.03 | 0.27±0.01 | 255.17±27.37 | 255.00±3.77 | 1.08±0.13 |
| 21 | 1 | 0.54±0.02* | 0.44±0.02* | 1011.1±45.5* | 473.00±17.39* | 3.13±0.31* |
| | 2 | 0.48±0.04* | 0.36±0.04* | 619.7±100.8*+ | 376.6±18.3*+ | 3.46±0.55* |
| | 3 | 0.52±0.02* | 0.44±0.03* | 823.90±54.04*+ | 277.0±18.6+ | 3.05±0.25* |
| | 4 | 0.47±0.05* | 0.36±0.03* | 575.8±113.3*+ | 307.2±12.3*+ | 3.40±0.12* |
| 40 | 1 | 0.29±0.02 | 0.38±0.01* | 155.30±15.07* | 545.33±21.56* | 5.69±0.35* |
| | 2 | 0.24±0.02 | 0.39±0.01* | 133.42±18.75* | 534.80±54.84* | 5.45±0.60* |
| | 3 | 0.25±0.01 | 0.34±0.01 | 98.92±5.27*+ | 418.67±15.44*+ | 3.79±0.26*+ |
| | 4 | 0.25±0.01 | 0.34±0.01 | 140.92±10.20* | 460.00±22.08*+ | 3.79±0.22*+ |

**Note.** AP, alkaline phosphatase; NCII, number of cells of the inflammatory infiltrate (per standard area of liver section); RACF, relative area of collagen fibers in the liver. *1*, Chronic toxic hepatitis; *2*, administration of IMHD in a daily dose of 35 U/kg for 2 days; *3*, administration of IMHD in a dose of 50 U/kg for 10 days at 1-day intervals; *4*, administration of IMHD in a dose of 35 U/kg for 10 days at 1-day intervals. $p<0.05$: *compared to the baseline (intact animals); +compared to the control (chronic hepatitis group).

in control specimens. No differences were found in the absolute and relative weight of the liver in rats of the control and treatment groups.

Biochemical study showed that ALT, AST, and alkaline phosphatase activities increased significantly in blood serum from animals of the control and treatment groups. However, alkaline phosphatase activity in blood serum from IMHD-treated rats was much lower than in animals of the saline group (Table 1).

Histological study of liver specimens after staining with hematoxylin-eosin revealed impairment of the lobular structure in control animals. The fields of granulation tissue were shown to substitute dead hepatocytes. They were characterized by the formation of new vessels and hepatic ducts. We revealed a considerable number of Councilman's bodies. Large-drop fatty degeneration was pronounced in preserved hepatocytes. Fusion of some cells was followed by the formation of fatty cysts. Regenerative hypertrophy of liver cells and considerable number of mitotic hepatocytes were observed under these conditions. These changes became most pronounced by the 21st day. Administration of IMHD was followed by a significant decrease in the number of infiltrating cells (day 21) and area of connective tissue (as compared to the control, day 40; Table 1). However, small-drop fatty degeneration was also typical of hepatocytes in this period.

Biochemical and morphological examination of the liver in rats showed that the product of IMHD has hepatoprotective properties. This agent produced the anticholestatic (decrease in the concentration of alkaline phosphatase in blood serum), anti-inflammatory, and antisclerotic effects.

Previous studies at the Institute of Pharmacology (Siberian Division of the Russian Academy of Medical Sciences) showed that the product of native hyaluronidase is potent in stimulating the function of progenitor cells (*e.g.*, proliferative activity and mobilization properties) [6,8]. Taking into account these findings and published data, we studied the effect of IMHD on the pool of bone marrow, circulating, and regional (hepatic) precursor cells.

Administration of $CCl_4$ was followed by a significant decrease in the number of tissue-specific colony-forming cells in the liver. The amount of fibroblast

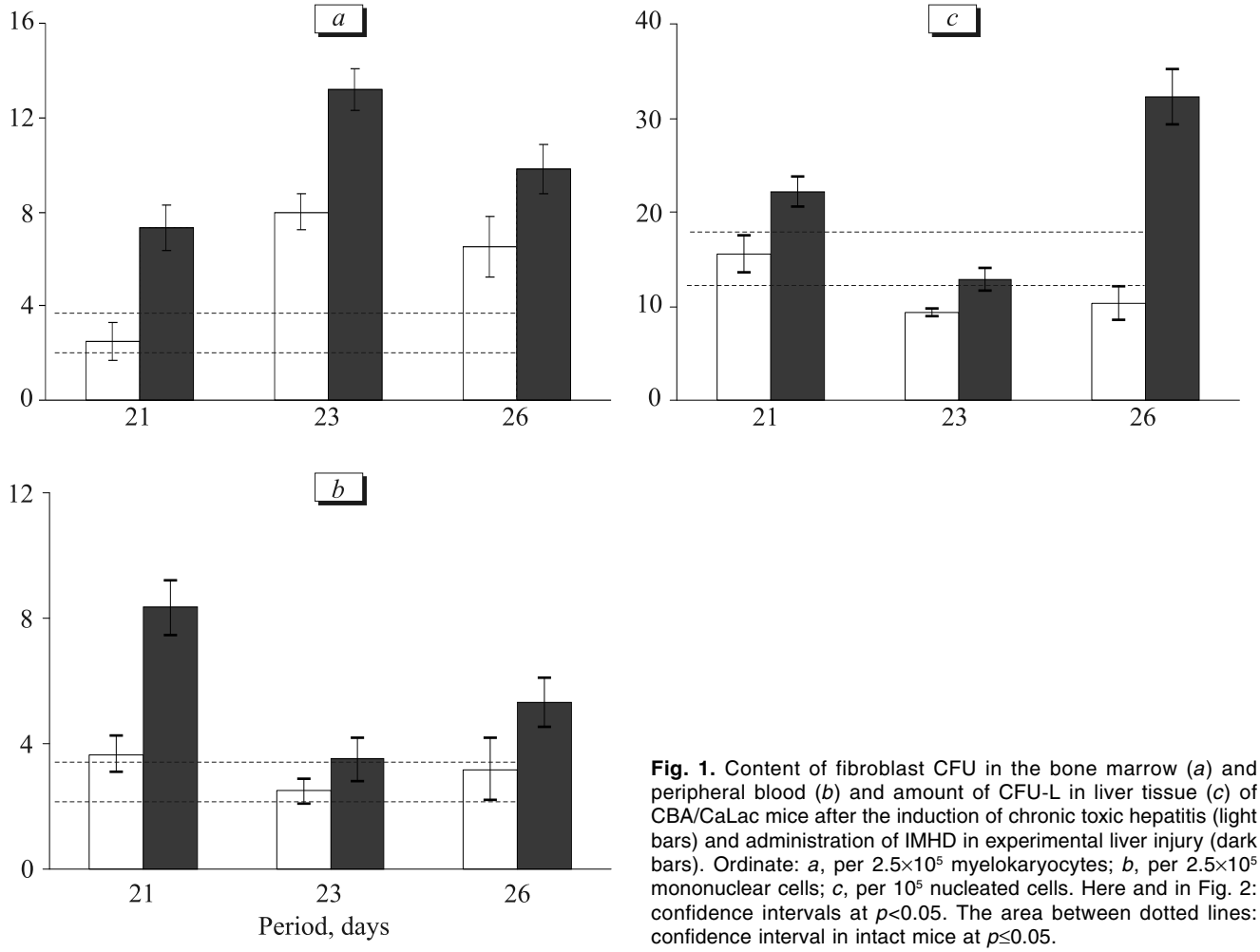


**Fig. 1.** Content of fibroblast CFU in the bone marrow (*a*) and peripheral blood (*b*) and amount of CFU-L in liver tissue (*c*) of CBA/CaLac mice after the induction of chronic toxic hepatitis (light bars) and administration of IMHD in experimental liver injury (dark bars). Ordinate: *a*, per $2.5\times10^5$ myelokaryocytes; *b*, per $2.5\times10^5$ mononuclear cells; *c*, per $10^5$ nucleated cells. Here and in Fig. 2: confidence intervals at $p<0.05$. The area between dotted lines: confidence interval in intact mice at $p\leq0.05$.

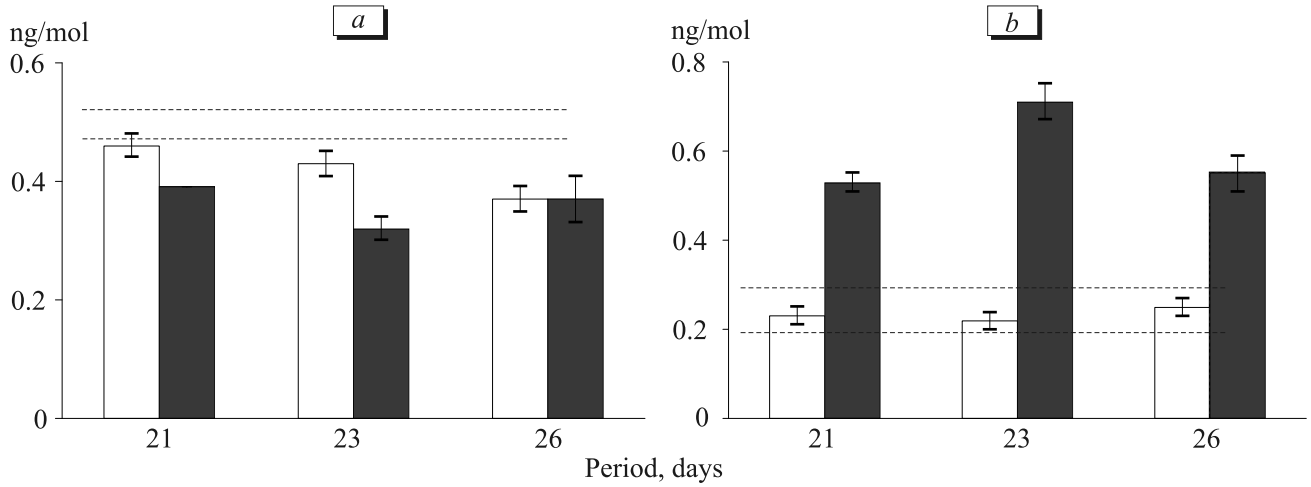


**Fig. 2.** SDF-1 content in conditioned media from adherent bone marrow cells (*a*) and liver cells (*b*) of CBA/CaLac mice after the induction of chronic toxic hepatitis (light bars) and administration of IMHD in experimental liver injury (dark bars).

CFU in the bone marrow increased under these conditions. These structures include not only committed stromal precursors, but also true/multipotent stem cells [2,11] (Fig. 1). The former changes were probably related to the toxic effect of $CCl_4$ on regional stem cells of the target organ [11]. The latter changes were associated with stimulation of bone marrow stem cells ("deep reserve" of regeneration [2]) due to activation of the stress-realizing systems. However, accumulation of stem cells in the depot tissue [2,9] was not accompanied by their release into the peripheral blood (Fig. 1).

Hence, one of the most effective mechanisms for regeneration (migration of stem cells from reserve sources) was not realized. These data are consistent with the results of our previous experiments showing inconsistency of "deep reserve" compensation mechanisms during chronic toxic hepatitis [11].

IMHD caused a more significant and rapid increase in the number of fibroblast CFU in the bone marrow (up to 293.2, 164.6, and 151.2% of the control on days 21, 23, and 26, respectively). These changes accompanied a sharp increase in the count of mesenchymal precursor cells in the peripheral blood. The number of these cells reached maximum (294.3% of the baseline) on day 21. The increase in the amount of CFU-L in the liver was most significant by the end of observations (day 26, up to 311.4% as compared to control mice; Fig. 2).

The observed changes are probably related to activation of early precursor proliferation in the storage tissue, mobilization of these cells, and directional homing to the damaged liver [2,9].

SDF-1 is a factor of stem cell migration. SDF-1 is produced by stromal cells of various tissues and provides the taxis and homing of cells, which *in situ* bind to this substance via specific CXCR4 receptors along the concentration gradient [12].

We evaluated the effect of the test preparation on SDF-1 production by cells of the bone marrow microenvironment and liver tissue.

The toxic agent produced a significant and progressive decrease in the content of SDF-1 in conditioned media from adherent bone marrow cells, but had no effect on the production of this factor by liver cells (Fig. 2). The latter fact probably contributes to insufficiency of the compensatory mechanisms for the disorder that are associated with the regenerative potential of stem cells in storage tissues [2,9].

Administration of IMHD was followed by a more significant decrease in the production of SDF-1 by myelokaryocytes (as compared to the control group). By contrast, SDF-1 production by microenvironmental cells of the target organ was shown to increase sharply under these conditions (up to 230.4, 322.7, and 220% of the control on days 21, 23, and 26, respectively).

We conclude that IMHD has a strong hepatoprotective effect. Therapeutic activity of this product is probably related to functional stimulation of bone marrow stem cells, migration of these cells into the target organ, and realization of the growth potential in tissue-specific or microenvironmental cells. These cells mediate acceleration of reparative processes [2,6, 9]. It should be emphasized that IMHD produces selective and opposite effects on SDF-1-producing cells of the depot tissue and target organ. Previous studies showed that enzyme treatment is followed by an increase in adhesive properties of stem cells [3]. Our results and published data illustrate high activity of migration processes that are associated with the humoral and cellular mechanisms. These mechanisms determine the highest degree of stem cell migration.

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