Stem Cells in Experimental Chronic Hepatitis

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We studied the state of the pools of mesenchymal precursor cells in the bone marrow and peripheral blood and the dynamics of regional precursor cells in the liver in experimental chronic hepatitis. We showed that bone marrow mesenchymal stem cells are involved in liver regeneration in its chronic disease. Our results suggest that these mechanisms are insufficient for the recovery of liver tissue.

Key Words: *stem cells; chronic hepatitis*

Chronic hepatitis is one of the most prevalent and severe stubborn diseases of the liver, when the regeneration potential of this organ is often insufficient, which leads to development of cirrhosis.

The liver tissue usually recovers after injury at the expense of regional precursor cells (so-called Ito's cells) [7]. New data indicate that many tissues of the body contain mesenchymal stem cells (MSC) migrating into the focus of injury and giving rise to functionally active hepatocytes [4,10]. The mechanisms of chronic hepatitis formation and the regeneration potential of the liver in this disease, as well as the role of precursor cells of different degree of maturity in the regeneration processes remain poorly studied.

We studied the state of bone marrow and circulating pools of mesenchymal precursor cells of different degree of maturity and regional hepatic stem cells in experimental chronic hepatitis.

MATERIALS AND METHODS

Experiments were carried out on 2-month-old outbred rats (n=40) and CBA/CaLac mice (n=96) of both sexes (1st category conventional mice from Breeding Center of Institute of Pharmacology).

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Chronic CCl_4-induced hepatitis served as the experimental model of liver disease. The rats received 50% CCl_4 in olive oil (2 ml/kg) and the mice received 20% CCl_4 (10 ml/kg) for 3 weeks (twice weekly; a total of 6 doses) through a gastric tube.

Serum levels of aspartate and alanine aminotransferases (AST and ALT, respectively) and alkaline phosphatase were measured in rats on days 7, 14, 21, 28, and 40 after administration of CCl_4. Morphological examination included evaluation of the relative area of infiltration [1] and connective tissue on histological preparations of the liver stained with hematoxylin and eosin and after Van Gieson, respectively. The relative area of the connective tissue was evaluated by computer analysis of graphical data.

Binding of mesenchymal precursors by bone marrow microenvironment cells [3], the content of fibroblast CFU and MSC in the bone marrow and peripheral blood, and the counts of hepatic precursor cells were measured in experimental and control mice on days 3, 7, 10, and 14 after the last dose of CCl_4.

The number of fibroblast CFU in the bone marrow tissue and peripheral blood was evaluated by *in vitro* cloning in a semisolid methylcellulose culture medium with L-glutamine. The fraction of fibroblast CFU includes not only stromal precursor cells, but also MSC [8]. The cells were cultured for 7

days in a CO_2 incubator, after which the colonies were counted under an inverted microscope. Cell aggregations consisting of at least 50 spindle-shaped and stellate cells were considered as fibroblast colonies.

The content of MSC in the bone marrow (at all terms of the study) and peripheral blood (on day 3 after the last dose of CCl_4) was evaluated by the method of limiting dilutions [6] in our modification. To this end, the cells (peripheral blood myelokaryocytes and mononuclears) in different dilutions (maximum cell concentration 750,000/ml, minimum 2500/ml) were cultured in plastic plates for 6 weeks in a CO_2 incubator. The medium contained L-glutamine, heparin, and fibroblast growth factor (FGF-basic) and was replaced twice a week. Fibroblast-like cells were counted in each well after incubation. If their number was >10, the well was considered "positive", if <10, the well was considered "negative".

The content of hepatic precursor cells (CFU) was studied by hepatic tissue cloning in culture medium with L-glutamine, heparin, porcine multicomponent insulin, and stem cell factor. The material was cultured for 10 days in a CO_2 incubator, after which the colonies were counted under an inverted microscope. Round or irregularly-shaped formations containing more than 30 cells were considered as colonies.

The data were processed by variation statistics method using Student's *t* test and nonparametric Wilcoxon—Mann—Whitney *U* test. The incidence of MSC in the bone marrow and peripheral blood was evaluated using correlated linear model for Poisson distribution [6,7].

RESULTS

Rat deaths in the experimental group started after the 3rd dose of CCl_4. By the end of the study, 21.2% animals died. Pathomorphological study of the liver from dead animals showed macroscopic signs of fatty degeneration (the organ was enlarged, had abnormal color, clay-like consistency, and necrotic foci).

Biochemical analysis of the serum showed elevated (*vs.* control group) activities of ALT on days 7, 14, 21 and of AST on days 7 and 14 of the experiment. Liver involvement in experimental animals led to elevation of serum alkaline phosphatase on days 7, 14, and 21 of the experiment (Table 1).

Histological study of liver preparations from animals treated with CCl_4 on day 21 of the experiment showed large-droplet fatty degeneration, hepatocyte necrosis, development of cell infiltration in the liver parenchyma (mainly with macrophages and lymphocytes) of different severity, fields of granulation tissue replacing dead cells, and regeneration hypertrophy of hepatocytes. Infiltration was diffuse. The relative areas of infiltration and connective tissue significantly surpassed the corresponding parameters in healthy controls (Table 1).

On day 40 of the experiment morphological signs of hepatitis were less pronounced than on day 21. Fatty degeneration of hepatocytes persisted, but was small-droplet. The relative area of liver parenchyma infiltration (and hence, activity of inflammatory process) decreased to 15.4±1.2%, but remained significantly higher than in healthy animals. Similar shifts were observed for connective tissue area: on day 40 the connective tissue occupied 2.75±0.42% area, which was significantly less than on day 21 of the experiment, but more than in healthy animals (Table 1).

Cell culture studies showed increased counts of MSC and fibroblast CFU in the bone marrow on days 7 and 10 of the experiment. This was presumably caused by activation of mechanisms of deep reserves of regeneration, the next component of which should be mobilization of committed and primitive stem cells into the blood (Fig. 1). Indeed, the levels of fibroblast CFU in the peripheral blood increased on days 7, 10, and 14, while the count of MSC in the peripheral blood reached 63±10 cells

TABLE 1. Dynamics of Biochemical Parameters of Peripheral Blood and Morphology of the Liver in CBA/CaLac Mice with Experimental Toxic Hepatitis ($X \pm m$)

Parameter	Control	Day after CCl_4 treatment				
		7	14	21	28	40
ALT, μcat/liter	0.72±0.05	2.78±0.09*	2.80±0.07*	1.22±0.07*	0.45±0.02*	0.72±0.02
AST, μcat/liter	0.76±0.06	3.10±0.35*	2.43±0.09*	0.71±0.08	0.40±0.01*	0.71±0.03
Alkaline phosphatase, U/liter	447.15±42.35	947.4±97.7*	1283.1±172.6*	1262.4±137.3*	407.70±8.45	554.2±65.3
Infiltrate area, %	7.36±0.44			25.48±3.67*		15.4±1.2*
Connective tissue area, %	0.70±0.11			7.53±1.61*		2.75±0.42*

Note. *$p<0.05$ compared to the control.

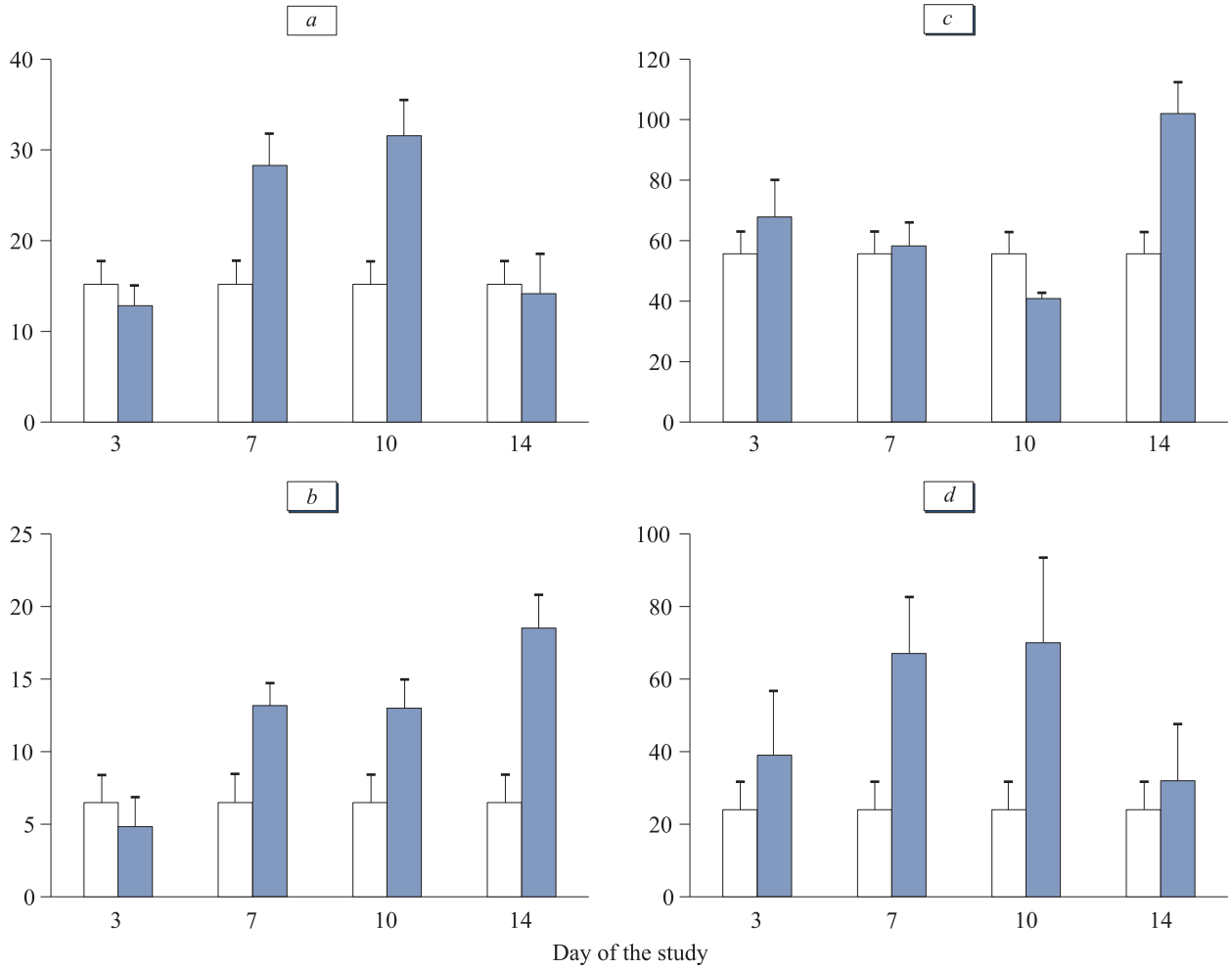

Fig. 1. The content of fibroblast CFU (per 250,000 myelokaryocytes) in the bone marrow (*a*) and fibroblast CFU (per 250,000 mononuclears) in the peripheral blood (*b*), CFU (per 10^5 nuclears) in the liver (*c*), and MSC count (per 10^6 myelokaryocytes) in the bone marrow (*d*) of intact CBA/CaLac mice (light bars) and animals with experimental chronic hepatitis (dark bars).

per 10^6 myelokaryocytes (*vs.* 31±7 cells/10^6 myelokaryocytes in the control) as early as on day 3.

However, the content of hepatic precursors decreased significantly in the involved organ on day 10 of the experiment, which suggests that during the early terms the liver regenerates mainly at the expense of regional precursor cells. MSC homing into the involved liver developed by the end of the experiment, which was seen from increased number of actively proliferating cells in the liver by day 14 of observation.

Hence, chronic toxic involvement of the liver associated with exhaustion of the pool of its regional stem cells led to activation, mobilization, and, presumably, homing of MSC into the liver tissue. However, it should be kept in mind that subsequent restoration of parenchymatous precursor cell population could be a result of not only MSC differentiation into specialized hepatic precursors [4,9,11], but also regeneration of tissue microenvironment damaged by CCl_4 treatment. It is known that the microenvironment via regulatory effects can essentially modify functional activity of organ-specific cells *in situ* [2].

On the whole, the results indicate insufficiency and/or failure of mechanisms of stem cell deep compensation reserves for the regeneration of the liver by restitution under conditions of chronic exposure to hepatotropic toxins.

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