Neuroprotective Effects of Immobilized Granulocyte Colony-Stimulating Factor and Hyaluronidase

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Neuroprotective activity of immobilized granulocyte CSF (nanotechnology with electron-beam synthesis) and hyaluronidase was studied on the model of posthypoxic encephalopathy. Oral administration of immobilized granulocyte CSF had no effect on manifestations of posthypoxic psychoneurological disorders in animals. Combined treatment with immobilized granulocyte CSF and hyaluronidase prevented impairment of orientation and exploratory behavior and development of amnesia in mice with hypoxic injury.

Key Words: *encephalopathy; immobilized granulocyte colony-stimulating factor; hyaluronidase; hypoxia*

Dysfunction of CNS is a common complication of hypoxic injury. In addition to standard antihypoxants, cytokines hold much promise for the correction of posthypoxic encephalopathy [5,4,7]. Previous experiments showed that granulocyte CSF (G-CSF) has granulocytopoiesis-stimulating and antihypoxic properties [4,5]. G-CSF produced a protective effect and prevented apoptosis in neurons under conditions of experimental ischemic stroke [12,13]. Moreover, this cytokine plays a role in mobilization and migration of stem cells (SC) of various classes from tissue depots to CNS. The microenvironment of CNS contributes to SC differentiation into highly specialized cells [12,13]. The therapeutic use of G-CSF products is limited because they can cause serious side effects, are prepared from an expensive recombinant form of cytokines, and are administered only via the parenteral route (injections) [1,7,13,14].

The preparation of immobilized G-CSF (IG-CSF) serves as an alternative for recombinant human G-CSF. IG-CSF was obtained by the nanotechnology method of electron-beam synthesis on low-molecular-weight polyethylene oxide. This product has several advantages. Immobilization of drug molecules on low-molecular-weight carriers prevents the enzymatic degradation. It should be emphasized that oral conjugates retain pharmacological activity [8]. However, the pathway of nanoparticle permeation through the blood-brain barrier remains unknown. Moreover, the mechanism of their action on the brain is unclear. Therefore, none of the nanoproducts for correction of CNS disorders was subjected to clinical trials.

It is difficult to develop novel products with high bioavailability. This problem can be solved by using the substances that improve permeation of medicinal agents into tissues (*e.g.*, CNS). Published data show that hyaluronidase decreases the viscosity of hyaluronic acid and causes its degradation to glucosamine and glucuronic acid. These changes contribute to the increase in tissue permeability (*e.g.*, for pharmacological agents) [7].

Here we studied the neuroprotective effect of individual or combined treatment with G-CSF (nanotechnology with electron-beam synthesis) and hyaluroni-

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dase under conditions of experimental posthypoxic encephalopathy.

MATERIALS AND METHODS

Experiments were performed on 84 male CBA/CaLac mice (class I conventional strain) aging 2 months and weighing 18-20 g. The animals were obtained from the nursery of the Institute of Pharmacology. Posthypoxic encephalopathy was induced by hypoxia in a sealed chamber. The animals were maintained in individual sealed chambers (500 ml) until the terminal stage of hypoxia (agonal seizure or visual cessation of breathing for 10-15 sec). Then they were removed from the chamber. The mice were repeatedly placed in a sealed chamber 5-10 min after the restoration of breathing and maintained there until the development of agonal state (generalized seizure or cessation of breathing) [4,5]. Conditioned passive avoidance response (CPAR) in animals was elicited 2 h after the removal from the sealed chamber [2,3]. CPAR performance was tested 1, 2, and 3 weeks after the acquisition. The emotional reaction and orientation and exploratory behavior of mice in the open-field test were studied on days 3, 7, 14, and 21 after hypoxia [2,3].

Experimental animals were divided into the following groups: group 1, subcutaneous injections of G-CSF (Neupogen, Hoffman-la Roche) in a daily dose of 100 μg/kg for 5 days; group 2, oral administration of IG-CSF in a daily dose of 100 μg/kg for 10 days; group 3, intraperitoneal injections of hyaluronidase (Lidaza, Research and Production Company "Mikrogen") in 0.3 ml physiological saline for 2 days (daily dose 20 arb. units); group 4, intraperitoneal injections of hyaluronidase for 2 days+subcutaneous injections of G-CSF (after 1 h) for 5 days; and group 5, intraperitoneal injections of hyaluronidase for 2 days+oral administration of IG-CSF (after 1 h) for 10 days. An equivalent volume of physiological saline (0.2 ml) was administered to mice of the intact control group and hypoxic control group under similar conditions. The first treatment with both preparations of G-CSF and hyaluronidase in animals of various groups was performed immediately after hypoxia.

IG-CSF was developed at the "Scientific Futures Management" Company and Institute of Pharmacology (Siberian Division of the Russian Academy of Medical Sciences). The molecules of non-glycosylated G-CSF were immobilized on low-molecular-weight polyethylene oxide by means of electron-beam synthesis (directed flow of accelerated electrons) [12].

The results were analyzed by methods of variation statistics (parametric Student's *t* test and nonparametric Mann–Whitney test). Test parameters were expressed in fractions. An exact Fisher test was used to compare the fractions.

RESULTS

Studying the orientation and exploratory behavior of animals in the open field showed that total locomotor activity of intact mice decreases progressively on days 7, 14, and 21 (Table 1). The innate behavioral reaction of animals (orientation and exploratory activity) was stimulated in a novel environment of the open field. Published data show that this reaction occurs over the first minutes of open-field testing and is mainly related to the state of fear [2,3,9]. The significance of emotiogenic factors (unexpectedness, novelty, and uncommonness) is reduced in the repeated open-field test. Hence, locomotor activity of animals decreases progressively in each successive test.

Hypoxic injury was followed by serious psychoneurological disorders in animals. The emotional reaction and total locomotor activity of animals in the open field were reduced on day 3 after hypoxia (compared to the intact control; Table 1). Total locomotor activity of mice from the hypoxic control group increased sharply in the late stage after treatment (day 7) and did not decrease in the follow-up period (days 14 and 21; Table 1). Hypoxia modulated not only the reaction of habituation, but also conditioned response activity. This conclusion was derived from the increase in the latency of entrance into the dark compartment and impairment of CPAR retention on days 14 and 21 (compared to the intact control; Table 2). Manifestations of posthypoxic encephalopathy in animals of the hypoxic control group were observed up to the end of the study.

The reference preparation of G-CSF prevented the development of psychoneurological disorders after hypoxic injury (Tables 1 and 2). Total locomotor activity of G-CSF-receiving animals decreased progressively by the 21st day. The degree of CPAR retention was high in various periods of the experiment. Oral administration of IG-CSF had no effect on the manifestations of posthypoxic encephalopathy in mice (Tables 1 and 2).

Hyaluronidase was administered alone or in combination with IG-CSF and G-CSF. This treatment prevented the development of changes after hypoxic injury. Orientation and exploratory activity of animals in the open field was shown to decrease progressively to the 21st day (Table 1). CPAR testing revealed no changes in the conditioned response reaction (Table 2).

Study preparations had no effect on the emotional reaction of animals on day 3. However, the emotional reaction of mice was reduced by the 14th day after treatment with these preparations (except for IG-CSF).

It should be emphasized that hypoxic exposure was followed the death of some mice receiving hy-

TABLE 1. Effect of Hypoxic Injury on Orientation and Exploratory Behavior of CBA/CaLac Mice in the Open Field ($X \pm m$)

Posthypoxic period, group		Total locomotor activity	Horizontal activity	Vertical activity	Hole reflex	Grooming	Defecation rate
Day 3	intact control	43.7±3.3	15.2±1.6	2.0±0.3	25.8±2.3	0.6±0.2	0.1±0.1
	hypoxic control	34.8±2.8*	11.9±1.9	1.6±0.4	21.3±1.2*	0	0.1±0.1
	G-CSF	36.0±3.9	13.2±2.0	0.7±0.3*	21.5±2.3	0.3±0.2	0.3±0.2
	IG-CSF	40.8±6.9	15.3±2.9	1.8±0.4	23.2±4.1	0.3±0.2	0.3±0.1
	hyaluronidase	31.9±3.2*	10.7±2.1	1.0±0.2*	19.1±1.6*	0.8±0.3	0.3±0.2
	G-CSF+hyaluronidase	46.4±4.6	19.1±2.3	2.0±0.8	24.4±2.4	0.7±0.5	0.2±0.1
	IG-CSF+hyaluronidase	45.7±3.9	17.9±2.1	0.9±0.3*	26.5±2.3	0.0±0.1	0.4±0.1
Day 7	intact control	31.4±4.6$^+$	12.6±2.9	1.6±0.5	15.7±2.2$^+$	1.6±0.7	0.1±0.1
	hypoxic control	46.2±6.4$^+$	18.1±3.4$^+$	2.2±0.5	24.7±3.5	0.9±0.4$^+$	0.3±0.1
	G-CSF	25.8±3.2	8.7±3.4	1.0±0.4	14.2±3.9	1.5±0.4$^+$	0.4±0.2
	IG-CSF	52.9±8.6$^+$	22.1±4.2	2.6±0.7	27.8±4.4	0.5±0.3	0
	hyaluronidase	25.4±4.8	9.3±2.4	1.9±0.6	13.0±2.4$^+$	1.2±0.4	0
	G-CSF+hyaluronidase	29.3±5.9$^+$	10.6±2.8$^+$	2.0±0.3	13.8±3.3$^+$	2.4±0.3$^+$	0.6±0.3$^+$
	IG-CSF+hyaluronidase	40.3±4.6	17.4±3.9	1.2±0.4	20.6±3.8	1.1±0.4$^+$	0
Day 14	intact control	27.9±3.2$^+$	11.8±3.5	1.6±0.4	14.1±2.4$^+$	0.2±0.1	0.2±0.1
	hypoxic control	40.8±5.3	18.1±2.8	2.2±0.6	19.7±2.5	0.2±0.2	0.6±0.2$^+$
	G-CSF	21.5±4.7$^+$	8.0±2.2	1.1±0.4	11.1±2.2$^+$	1.1±0.4	0.2±0.1
	IG-CSF	37.0±4.9	17.0±4.8	1.9±0.8	17.5±4.6	0.4±0.2	0.4±0.2
	hyaluronidase	17.4±3.3$^+$	6.5±2.9	0.5±0.3	9.1±3.2$^+$	0.9±0.4	0.4±0.2
	G-CSF+hyaluronidase	25.2±4.2$^+$	12.6±2.2	1.7±0.7	9.4±1.9$^+$	1.3±0.5	0.2±0.1
	IG-CSF+hyaluronidase	25.3±3.0$^+$	12.6±3.6	1.4±0.4	9.8±2.3$^+$	1.3±0.4$^+$	0.2±0.1
Day 21	intact control	30.1±3.8$^+$	13.6±2.7	1.1±0.3	10.9±1.9$^+$	3.0±0.5$^+$	1.6±0.5$^+$
	hypoxic control	40.4±4.2	20.2±2.1$^+$	2.2±0.6	15.3±2.0$^+$	2.2±0.7$^+$	0.5±0.2$^+$
	G-CSF	21.8±4.6$^+$	8.1±2.7	1.5±0.4	10.4±1.7$^+$	1.5±0.3$^+$	0.3±0.1
	IG-CSF	40.1±2.9	21.5±1.6	1.7±0.6	15.2±1.6	1.4±0.4$^+$	0.4±0.1
	hyaluronidase	19.8±3.3$^+$	8.1±3.3	0.9±0.5	8.1±2.2$^+$	1.8±0.7$^+$	0.9±0.2$^+$
	G-CSF+hyaluronidase	27.7±4.7$^+$	11.0±2.6$^+$	12.8±0.5	11.8±1.7$^+$	2.4±0.4$^+$	0.7±0.2
	IG-CSF+hyaluronidase	25.6±4.8$^+$	12.7±3.1	1.3±0.4	10.1±1.7$^+$	1.3±0.4$^+$	0.3±0.1

Note. $p<0.05$: *compared to intact control; $^+$compared to day 3.

aluronidase. Moreover, these animals had low values of total locomotor activity in the open field and light compartment of the CPAR chamber. Studying the emotional reaction showed that muscle tension is low in hyaluronidase-receiving mice. Therefore, the influence of hyaluronidase cannot be considered as a positive effect.

Our results indicate that the reference preparation of G-CSF exhibits high antihypoxic activity. Oral administration of IG-CSF was ineffective during posthypoxic encephalopathy. However, combined treatment with IG-CSF and hyaluronidase prevented the development of psychoneurological disorders in animals. This effects is probably associated with the fact that hyaluronidase improves IG-CSF bioavailability due to an increase in blood-brain barrier permeability for this agent. It cannot be excluded that the improvement of higher nervous activity is related to the ability of this enzyme to potentiate the effect of IG-CSF on "deep" regeneration reserves (SC) [4].

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TABLE 2. Effect of Hypoxic Injury on CPAR Retention by CBA/CaLac Mice ($X \pm m$)

Group	LC, sec	Posthypoxic period, days		
		7	14	21
Intact control	55.1±7.1	100*	90*	100*
Hypoxic control	74.0±7.8	82	45	20
G-CSF	54.6±5.8	100*	80*	90*
IG-CSF	69.1±7.4	73	36	18
Hyaluronidase	71.5±7.1	90	88*	63*
G-CSF+hyaluronidase	73.3±7.9	100*	67*	67*
IG-CSF+hyaluronidase	71.3±9.8	91	64	64*

Note. LC, latency of entrance to the dark compartment during CPAR training. *$p<0.05$ compared to hypoxic control.

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