Cardiotropic Effect of Extracardiac Transplantation of Embryonic Human Myoblasts to Mice with Bradycardia: Various Effects of Cell Material

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Animals with bradycardia were detected in reproductive colony of mdx mice. Low pulse rate was associated with poor survival and predisposition to sudden death, but did not directly depend on the presence of dystrophin mutant gene or animal age. Heart rate increased in old mice with bradycardia after extracardial, intramuscular, and intravenous injection of human embryonic myoblasts. Stable normalization of the pulse was observed 2 weeks after transplantation, but early peak of heart rate was observed as early as 24 h after cell transplantation. Cell suspensions, which could contain stem cells (blood mononuclears and $CD34^+$ lymphocytes), also corrected heart rhythm. Unlike the effect of myoblasts, cardiotropic effect of mononuclears was preceded by a period of tachycardia, while the effect of $CD34^+$ lymphocytes was very unstable. The cardiotropic effect of myoblasts was combined with life span prolongation and certain "rejuvenation" in some animals. Erythrocytes and supernatant obtained during blood cell fractionation did not modify the heart rhythm in mice with bradycardia. After injection of myoblasts to mice with rare and normal pulses serum creatine kinase activity decreased with different rates. These data attest to a variety of biological effects of stem cells and/or their derivatives and to ambiguous mechanisms of these effects.

Key Words: *myoblasts; cell therapy; heart rhythm; mdx mice*

The idea of therapeutic use of stem cells and their derivatives for the repair of damaged tissues opens a new approach to the cure of patients heretofore considered incurable, for example, boys with Duchenne's progressive muscular dystrophy (PMD). Attempts at treating PMD by intramuscular injections of myogenic precursors (myoblasts) capable of fusing with recipient myofibrils and delivering dystrophin into them were described [6,9-11]. The absence of this protein in muscle fiber sarcolemma is determined by molecular defect in the dystrophin gene, which together with high activity of serum creatine kinase is regarded in clinical practice as the PMD "visiting card". It is known that stem cells can migrate to damaged organs and tissues distant from the site of injection [12]; reparation of the recipient tissue can be due to not only histogenesis of the transplanted cells up to incorporation of differentiated descendants from the donor stem cells into host cell ensembles, but also due to stimulation of host histogenesis by transplanted cells [7].

Human embryonic myoblasts injected into skeletal muscles of mdx mice with hereditary muscular dystrophy partially restored the expression of dystrophin protein in myofibrils [3]. Studies of the PMD cell therapy are mainly focused on reparative processes in skeletal muscles, while the involved heart [1,4] is neglected, though the effects of injected stem cells can be observed also in the heart.

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We analyzed the effects of human embryonic myoblasts transplanted to mdx mice for the treatment of muscular dystrophy on their cardiovascular system.

MATERIALS AND METHODS

The study was carried out on male mice of different ages typed and not typed for mutant dystrophin gene carriership by the DNA diagnosis method [3]. These mice were a part of the reproductive colony maintained at Russian State Medical University [2].

ECG in standard lead II was recorded on nonanesthesized alert animals in natural (abdominal) posture with the limbs slightly fixed to the platform. Needle electrodes for ECG recording were inserted in the forearms and shins. Heart rhythm, its rate, and duration of *R—R* intervals were studied. Because of variability of the *R—R* intervals, we calculated the mean heart rate for each mouse by the formula:

$$Y = \frac{\Sigma \frac{60{,}000}{R—R_i}}{N}$$

where Y is the mean heart rate (HR_{mean}, bpm) for each mouse, $R—R_i$ duration of a certain *R—R* interval (msec), and N number of measured *R—R* intervals.

Activity of creatine kinase (CPK) was measured in blood samples collected from the caudal vein as described previously [2].

Human embryonic myoblasts obtained as described previously [3] were injected intramuscularly or intravenously in a dose of 500,000 per mouse. Controls were intramuscularly injected with mononuclear cells (MNC) isolated from human blood; MNC suspension containing 60% $CD34^+$ cells; erythrocytes and supernatant obtained during fractionation of blood samples for MNC isolation. Spontaneous HR_{mean} fluctuations were recorded during a long period in a special group of control mice.

The statistical significance of changes in cardiac rhythm was evaluated using Student's parametric test, nonparametric sign test (ST), and inversion test.

RESULTS

Experimental model for studying the cardiotropic effect of myoblasts. The study of cardiac activity of

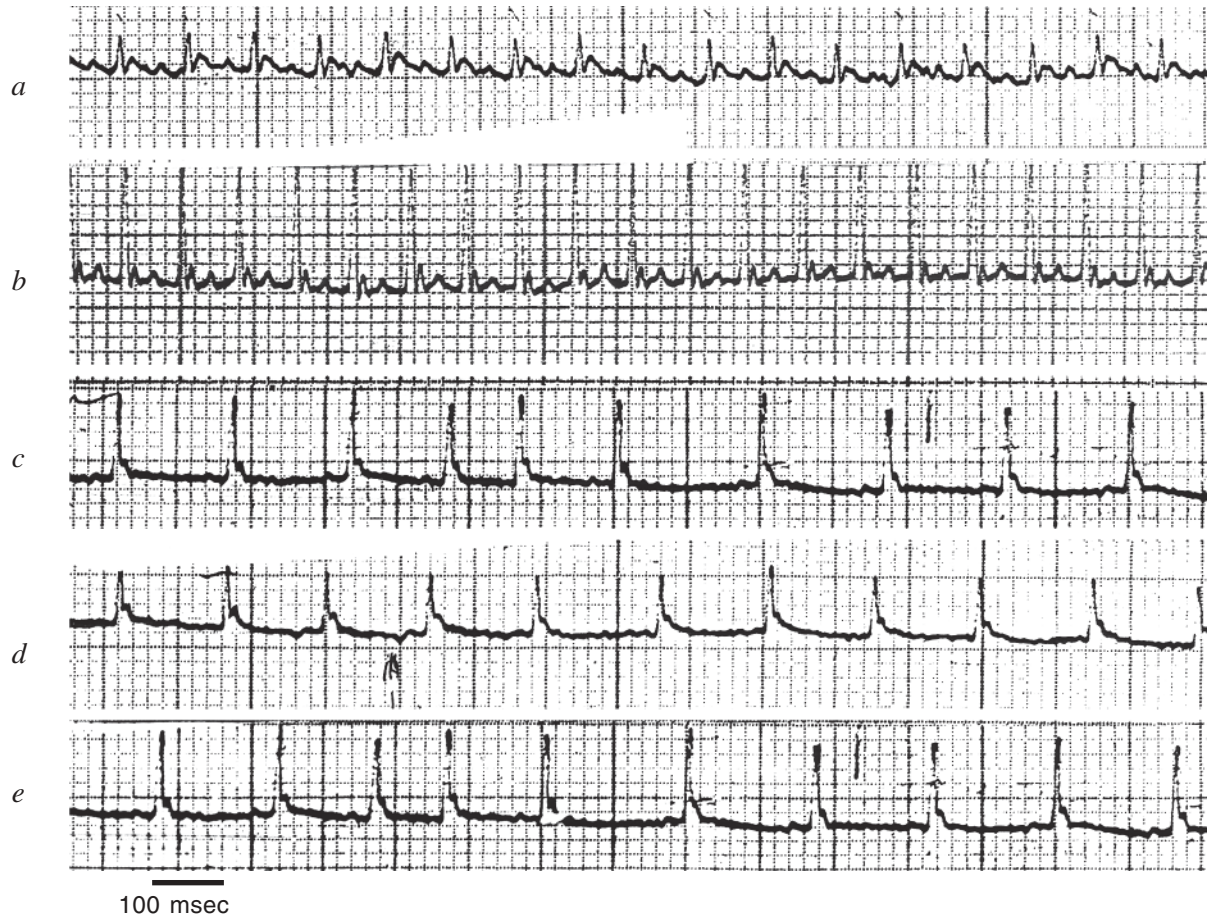

Fig. 1. ECG of 18-month-old mice with rare (*c-e*) and normal (*a*, *b*) pulse. The records were made in standard lead II, type rate 100 mm/sec, mV=2 cm.

mdx mice showed that the colony consisted of animals with normal (650-750 bpm) and rare (300-500 bpm) pulse. Pulsus rarus is a characteristic of mice selected by this sign, because bradycardia persisted in these animals during long-term observation.

ECG of mice with bradycardia (Fig. 1, *c-e*) showed, apart from increased *R—R* interval, other pathological shifts: decreased amplitude of *T* and *P* waves, up to complete disappearance of the latter in some cycles, which resulted in distortion of the sinus rhythm and transition to idioventricular rhythm. Lengthening of *P—Q* and *Q—R—S* intervals reflects decelerated intracardiac conduction. Deformation of *R* wave reflected dystrophic changes in the myocardium, while changes in the *S—T* interval indicated disorders in coronary circulation in mice with bradycardia.

The colony consisted of mice of several genotypes: normal (x/y males and x/x females) and mutant animals (mdx/y males and mdx/x heterozygous and mdx/mdx homozygous females). Analogous genotypes (except mdx/mdx) are present in human population. Screening of more than 200 mice showed that 20-25% mice of each genotype had pulsus rarus. Rare pulse does not seem to be directly related to mutant dystrophin gene carriership.

The percentage of mice with bradycardia was higher among old animals, though low HR_{mean} was observed in animals aged 4 and even 1.5 months.

Bradycardia is observed in mice of other strains related to mdx strain: C57Bl10/Snell, C57Bl/6, C57Bl10/Ks (db/db genotype). The detected defect of cardiac activity seems to be as prevalent in mice as in human population.

Cardiotropic effect of cell therapy. Figure 2, *a*, presents typical curves of changes in HR_{mean} after injection of myoblasts to untyped old mice with rare (n=10) and normal (n=5) pulse. Cardiac rhythm was analyzed during periods longer than the minimum period required for *in vivo* differentiation of myogenic precursors (more than 2 weeks) and during week 1 after transplantation (early changes in heart rhythm).

Stable normalization of the heart rhythm was observed 2 weeks after injection of myoblasts to mice with bradycardia; these animals were observed in experiment during at least 27 weeks (Fig. 2, *a*; subgroups 1a and 1b). The statistical significance of the effect was confirmed by comparison of the initial pulse rate and HR_{mean} during weeks 2, 4, 8, 10, 14, 18, 23, 27, and 36 of the experiment (p<0.05, ST).

The mean pulse surpassed 600 bpm in animals of subgroups 1a and 1b and approached this level in 2 mice, which died during the first 2 weeks of the experiment (subgroup 1c).

Heart rhythm normalized irrespective of the route of myoblast injection (intramuscular or intravenous).

Cell suspensions potentially containing stem elements (MNC, n=3, and $CD34^+$ lymphocytes, n=3) modified HR_{mean} in mice with bradycardia similarly as myoblasts. However, after injection of MNC HR_{mean} stabilized after a period of pronounced tachycardia.

The effect of $CD34^+$ lymphocytes (normalization of the heart rhythm) was less lasting than the effects of myoblasts and MNC.

Injections of erythrocytes or supernatant to mice with pulsus rarus (n=6) did not increase HR_{mean}. This parameter varied within the range characteristic of mice with bradycardia.

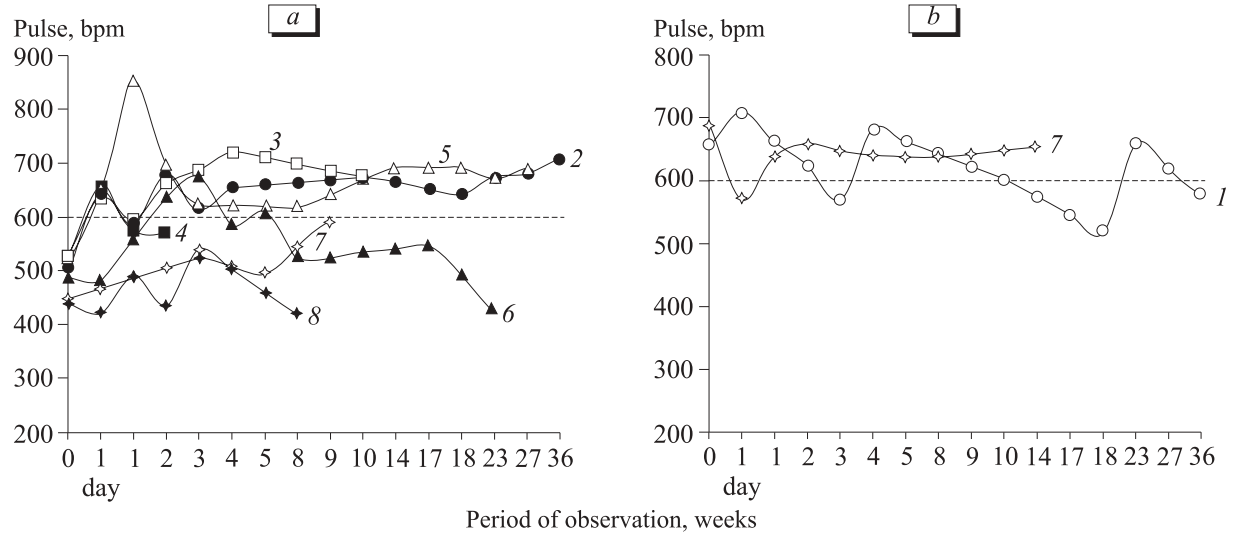

Fig. 2. Changes in the heart rhythm in old mice with rare (*a*) and normal (*b*) pulse after cell therapy. *1-4*) human embryonic myoblasts; *5*) blood mononuclear cells; *6*) $CD34^+$ cell-enriched suspension; *7*) erythrocytes; *8*) supernatant obtained after blood cell fractionation. Life span in the experiment surpassed 23 weeks (*2*; subgroup 1a), 10 weeks (*3*; subgroup 1b), was no more than 3 weeks (*4*; subgroup 1c). Transplantation effect was monitored over 36 weeks.

TABLE 1. Serum CPK Activity and Cardiotropic Effect of Cell Therapy in Mutant mdx Males

Group	HR_{mean}, bpm			Creatine kinase, U/liter			
	initial	1 month	4 months	initial	1 month	2 month	4 months
Normal pulse (n=10)	669	672	675	2105	302**	469	834
Pulsus rarus (n=6)	511	622*	636*	1881	982*	603**+	1781

Note. *p<0.05, **p<0.01 compared to initial level (nonparametric test of signs), +p<0.005 compared to the group with normal pulse.

No cardiotropic effect of cell material was observed in mice with normal pulse (Fig. 2, *b*).

The effects of skeletal myoblasts on cardiac contractile function was demonstrated in clinical studies (the cells were injected directly into the myocardial infarction zone) [8]. In our experiments heart rhythm in mice with bradycardia was stabilized by myoblasts injected extracardially (into the blood or limb muscles).

Y. Torrente *et al.* [12] detected donor stem cells soon after their intramuscular injection to mdx/mdx mice in other groups of muscles, blood, and bone marrow; this experiment demonstrated migration capacity of stem cells. Presumably, embryonic myoblasts used in our experiments contained an admixture of stem cells. The data on myoblast migration mainly concern their migration in muscle tissue. We found no reports comparing the migration capacity of muscle stem cells and myoblasts from tissues into the blood.

According to some data [7], skeletal myoblasts do not incorporate into the cell ensemble of cardiomyocytes in the myocardium, and the therapeutic effect of cardioplasty can be explained by alternative mechanisms, for example, stimulation of endogenous pool of stem cells by transplanted cells [7]. Presumably, extracardially injected myoblasts trigger these mechanisms through soluble factors. This hypothesis is based on the data of gene engineering studies demonstrating that myoblasts fusing with multinuclear cells (microtubes) form a chronic focus in tissues releasing into the blood the products of genes incorporated in DNA, *e.g.* clotting factor IX, during many months [13].

Early changes in cardiac rhythm. Characteristic changes in HR_{mean} were observed during the first week after injection of myoblasts to mice with rare and normal pulses: acceleration of cardiac rhythm compared to the initial level as early as after 24 h, and its deceleration by the end of the week (p<0.01, ST). The curve representing the dynamics of HR_{mean} changes after myoblast injection shows an early peak of the parameter. No early changes in cardiac rhythm were observed after transplantation of MNC, $CD34^+$ lymphocytes, erythrocytes, or supernatant (Fig. 2). Massive death of myoblasts during the first days after transplantation into the muscle [5] suggests that the spectrum of embryonic cell degradation products includes compounds exerting an early distant unstable effect, presumably due to the influence on the autonomic nervous system.

Cell therapy and stimulation of mouse viability. According to our observations, pulsus rarus in mice was associated with sudden death during virtually atraumatic manipulations: blood collection, studies of motor activity, *etc.* Some animals died in experimentator's hands during examination. Adult mice with pulsus rarus weighed 20-25 g (normal weight 30-40 g).

The life span of mice selected by pulsus rarus was lower than that of mice with normal pulse. In a special experimental series we evaluated viability of 18-month-old mice with pulsus rarus (470±34 bpm) in comparison with control animals (671±24 bpm). All 10 mice with normal pulse were alive 7 months after the start of the experiment. Mean heart rate did not change in this group (683±20 bpm). All 14 mice with pulsus rarus died within the first 3 months.

Only mice injected with myoblasts survived during at least 36 weeks in the experiment (Fig. 2, *a*). The mice injected with erythrocytes or supernatant died much earlier. None of the animals injected with MNC or $CD34^+$ cells survived longer than 36 weeks. Hence, of the cell suspensions correcting to this or that measure the heart rhythm only embryonic myoblasts prolonged the life span of mice.

The physical status of some mice with pulsus rarus, which survived a long time after injection of myoblasts, improved: motor activity increased, dark hairs appeared in the foci of alopecia on the back. Correlation analysis of the data in the group of old mice indicated that the decrease in heart rate was paralleled by changes in blood biochemical constants up to acidosis: pH decreased (r=0.7; p<0.001) and partial carbon monoxide pressure increased (r=-0.07; p<0.002). Hence, processes directly not related to differentiation of myogenic precursors in muscles seem to underlie the "rejuvenating" effect of skeletal myoblasts.

Heart rate and specific response of mutant mdx males to cell therapy. The data (Table 1) show a correlation between the decrease in CPK activity as a specific effect of cells therapy, associated with the muscle system status, and the cardiotropic effect of this therapy. One month after injection of cells to mu-

tant mdx males aged 1.5 years homogeneous by the genotype the animals with normal pulse responded to therapy by a more pronounced drop in CPK activity than mice with bradycardia. This result necessitates a differentiated approach to prescription of cell therapy to PMD patients, in whom myocardial involvement (for example, dilatation cardiomyopathy) is often concomitant with degenerative processes in skeletal muscles [1,4].

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