EXPERIMENTAL BIOLOGY

Xenotransplantation of Embryonic Precursors of Human Myogenesis for the Correction of Dystrophinopathy in Mice with Hereditary Muscular Dystrophy

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Human embryonic myogenic precursors were transplanted into muscles of mdx mice with hereditary dystrophin-deficient muscular dystrophy. Transplantation induced the synthesis of human dystrophin. The number of dystrophin-positive fibers progressively decreased, however, some of them were preserved even 5 months after transplantation. Our results indicate that xenogeneic transplantation of embryonic myogenic precursors compensates the genetic defect in dystrophin-deficient mice.

Key Words: *dystrophin; human myogenic precursors; transplantation; mdx mice*

Considerable attention is now given to the problem of stem cell transplantation because it holds much promise for the therapy of various diseases. Duchenne's muscular dystrophy is one of the most severe and common hereditary human diseases. The search for new methods for the therapy of this disease is in progress. Cell therapy with myoblasts from healthy donors performed under the control of immunosuppression can be used for temporal compensation of dystrophy. The studies in this field became recently more extensive because of the use of cells obtained at various stages of postnatal ontogeny [2,9,11,15]. After transplantation donor cells interact with muscle fibers in the recipients and with each other giving rise to new muscle fibers [10]. Published data show that this therapy produces only a short-term effect, because most cells die over the first few hours after transplantation. Some authors reported that only small number of cells survives in recipient tissue 1 week after transplantation [7]. However, according to other authorities activity of donor genes can be detected even 6 months after treatment. Suppression of the host immune response with various immunosuppressors, including FK 506 [8] and cyclosporine A [3,14], plays a critical role in this process. The advantages of treatment with stem and pluripotent embryonic cells are plasticity and high differentiation potential [4,7]. Therefore, these cells hold much promise for the therapy of Duchenne's muscular dystrophy and other diseases.

We previously elaborated new methodical approaches to the use of myogenic precursors for the therapy of muscular dystrophies [1]. It was interesting to

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evaluate whether cultured myogenic precursors (embryonic myoblasts obtained from abortion material on the 10th week of gestation) can be used to compensate dystrophin deficiency in mdx mice that serve as a biological model of Duchenne muscular dystrophy. We found no published data on the effect of transplantation of embryonic myoblasts in humans. Individual attempts were made to perform these experiments on animals. Japanese scientists conducted experiments with intraarterial administration of CD34 and Sca-1 cells from muscles of newborn mice. These cells migrated from the circulation into muscles of the recipient mice and were involved in the formation of myofibrils. Dystrophin synthesis was restored after this treatment [13]. In our study the choice of cells was determined by the possibility to isolate embryonic tissues of fetuses obtained from healthy women during abortions. We selected the gestational period when termination of pregnancy occurs most frequently.

We evaluated the ability of these cells to function in muscle tissue of the recipients, estimated their differentiation potential and duration of survival in host tissue, and analyzed the ability of cells to produce genetic transformation of dystrophin-deficient fibers in skeletal muscles of mdx mice. In addition, immunogenicity of these cells was evaluated without immunosuppressor treatment.

MATERIALS AND METHODS

Experiments were performed on 4-month-old males carrying mutations in exon 23 of the dystrophin gene. DNA assay was used to select animals from homozygotes and heterozygotes carrying mutations. Normal and mutant mice not transplanted with myoblasts served as the control.

Polymerase chain reaction (PCR) was performed by the standard method using specific primers MDX-R (5'-CTCAATCTCTTCAAATTCTGACAGA-3') and MDX-F (5'-CTCTGCAAAGTTCTTTGAAAGATTAA-3'). The reaction was conducted in an automatic amplifier (20 μl) containing 0.1 μg isolated DNA, 5 mM $MgCl_2$, 0.2 mM dNTP in PCR buffer, and 0.5 U Taq-polymerase. After primary denaturation at 95°C for 3 min 30 cycles (1 min at 94°C, 1.5 min at 50°C, and 1 min at 72°C) were carried out. After the last cycle the test samples were maintained at 72°C for 5 min. PCR products were analyzed by gel electrophoresis in 8% polyacrylamide gel (PAAG). Restriction assay was performed using restrictase VspI in 20 μl reaction mixture containing 15 μl amplifier and 5 U restrictase in 1× restriction buffer.

DNA fragments were separated using PAAG prepared from 8% acrylamide and N,N'-methylene-bis-acrylamide (ratio 1:30), Tris-borate buffer (0.089 M Tris, 0.089 M boric acid, and 0.002 M EDTA, pH 8.0), 0.07% ammonium persulfate, and tetramethylethylenediamine (dilution 1:3000). Electrophoresis was conducted in vertical plates (150×200×1 mm) at a constant voltage of 400 V for 1 h. The gel was stained with ethidium bromide and assayed in UV light.

The cell culture (donor material) was isolated from striated muscles of human fetuses obtained from clinically healthy women immediately after therapeutic abortion (10 weeks' gestation). Muscle tissue was washed in phosphate buffered physiological saline (PBS, pH 7.2, Sigma) containing 50 U/ml penicillin and 10 μg/ml streptomycin. The material was mechanically minced (<1.5×1.5 mm particles) in a sterile Petri dish (diameter 35 mm, Corning). These particles were placed in centrifuge tubes, washed 2 times with PBS, and centrifuged at 600*g* for 3 min. The tissue was dissociated with 0.2% type XI collagenase (Sigma) at 37°C for 20 min and treated with 0.1% solution of trypsin and EDTA (Sigma) for 15 min to obtain the single cell suspension. The population of cells (2×10^6/ml) was cultured in DMEM/F12 1:1 medium (Gibco) with 15% fetal bovine serum at 37°C and 5% CO_2 for 21 days. The medium was replaced at 3-day intervals. Hoechst 33342 in a final concentration of 20 μg/ml was added to the culture medium before transplantation. The cells were incubated in a CO_2 incubator for 1 h, washed 3-4 times in PBS, and diluted with physiological saline to a concentration of 4×10^6/ml.

Donor cells (500×10^3) in 250 μl sterile apyrogenic physiological saline were implanted into the central region of thigh muscles in the right limb. Physiological saline in an equivalent volume was administered into thigh muscles of the left limb. Injections were made using an insulin syringe under sterile conditions without anesthesia.

Chimerism and localization of transplanted human cells in tissues of the recipient mice were determined by staining for human nuclei. Some mice were narcotized with urethane in a lethal dose on day 3 or 7 after transplantation. Thigh muscles on the side of trans-

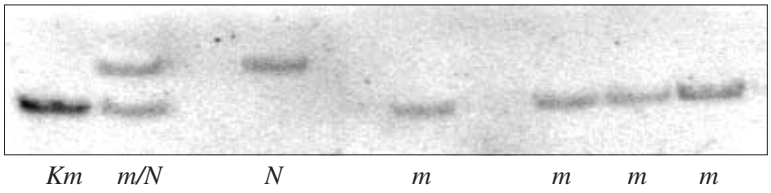

Fig. 1. Electrophoretic assay of restriction of exon 23 in the dystrophin gene of mdx mice. *Km*, control mouse with mutation in exon 23; m/N, heterozygous female; *m*, mutant mice.

plantation were dissected and fixed in 10% formalin and 30% sucrose for 12 h. Serial sections (40 μ) were prepared, stained with monoclonal antibodies to human nuclear antigen, treated with fluorescent dye-labeled secondary antibodies, and examined under a fluorescence microscope.

Muscle tissue from the recipient mice was subjected to immunohistochemical assay for the presence of dystrophin 1, 3, and 5 months after transplantation. We used antibodies to dystrophin epitopes DYS-1, DYS-2, and DYS-3 (Novocastra). Sections of non-fixed muscles (6 μ) were prepared on a cryostat. Endogenous peroxidase was inhibited with 0.6% H_2O_2 in PBS for 15 min. The sections were treated with primary antibodies DYS-1, DYS-2, and DYS-3 (dilution 1:10, 25 μl) at 25°C for 1 h, washed 3 times in 1× PBS for 5 min, incubated with secondary antibodies (goat anti-mouse peroxidase-conjugated antibodies, 25 μl, dilution 1:100) at 25°C for 1 h, washed 3 times in PBS for 5 min, and developed with 3.3'-diaminobenzidine tetrahydrochloride and 0.1% H_2O_2 for 4 min. Stained sections were examined under a light microscope.

RESULTS

The mice with specified genotypes (mutant animals carrying mutations in exon 23 of the dystrophin gene and normal wild-type males) were selected by DNA assay (Fig. 1).

Human embryonic myoblasts (500×10^3) obtained from fetuses at 10 weeks' gestation were intramuscularly transplanted to 4-month-old mutant males. The animals were under constant observation. We revealed no complications after transplantation of the cell suspension. Our results contradict to published data that most myoblasts die over the first hours after transplantation [9]. A considerable number of human myoblasts were viable, did not undergo apoptosis, and migrated from the site of injection (Fig. 2).

The mice were killed 1, 3, and 5 months after transplantation to evaluate whether donor cells can survive in tissues and compensate the genetic defect. Muscles of normal, mutant, and recipient mutant mice were assayed for the presence of dystrophin (Fig. 3, *a*, *b*). Experiments with NCL-DYS1 and NCL-DYS2 antibodies did not reveal dystrophin in mutant males,

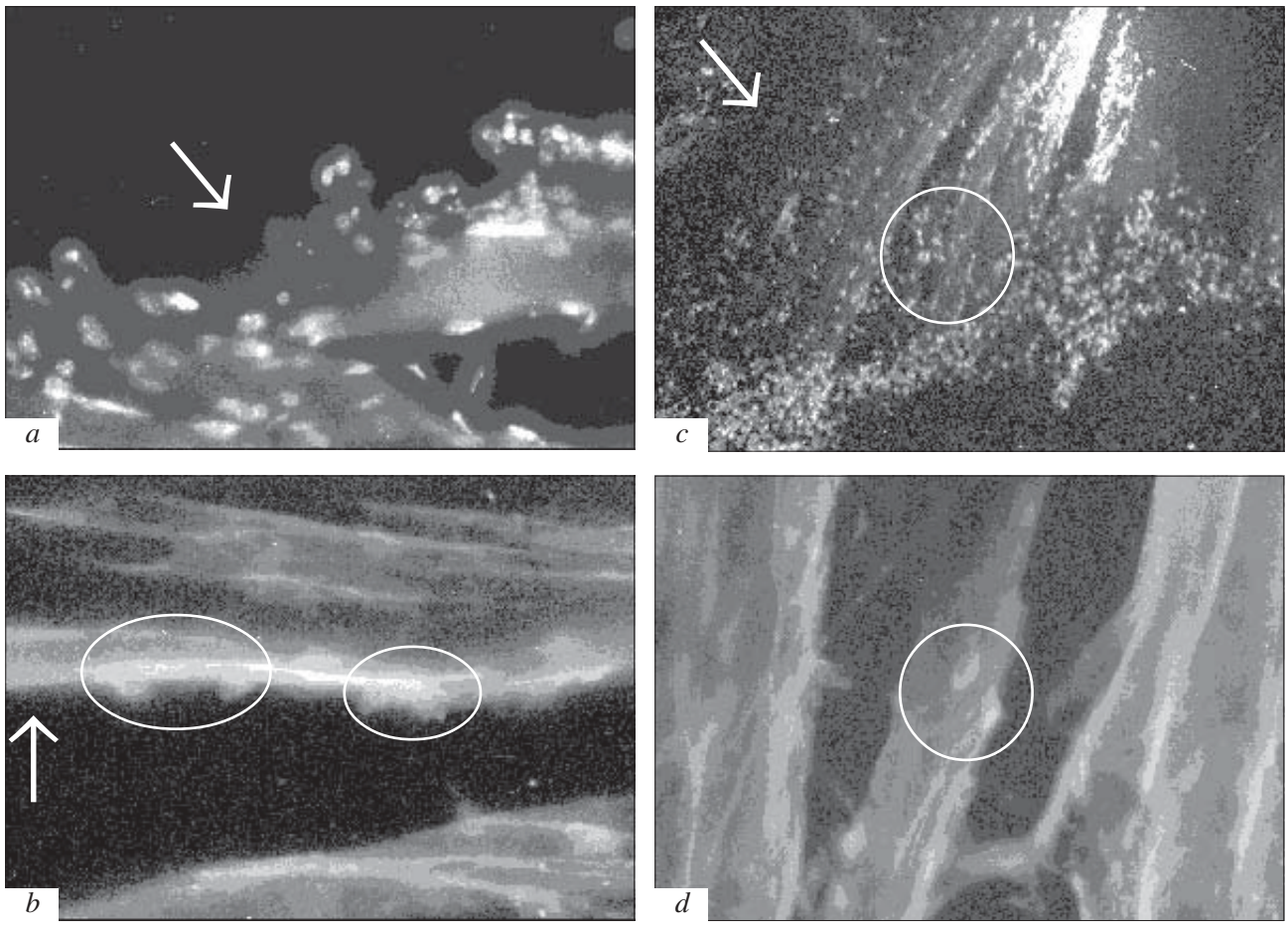

Fig. 2. Immunohistochemical staining of muscle tissue in recipient mice for human nuclei: 3 (*a-c*) and 7 days after transplantation (*d*). Arrow: muscle fibers. Circles: nuclei of transplanted myoblasts bound to muscle fibers.

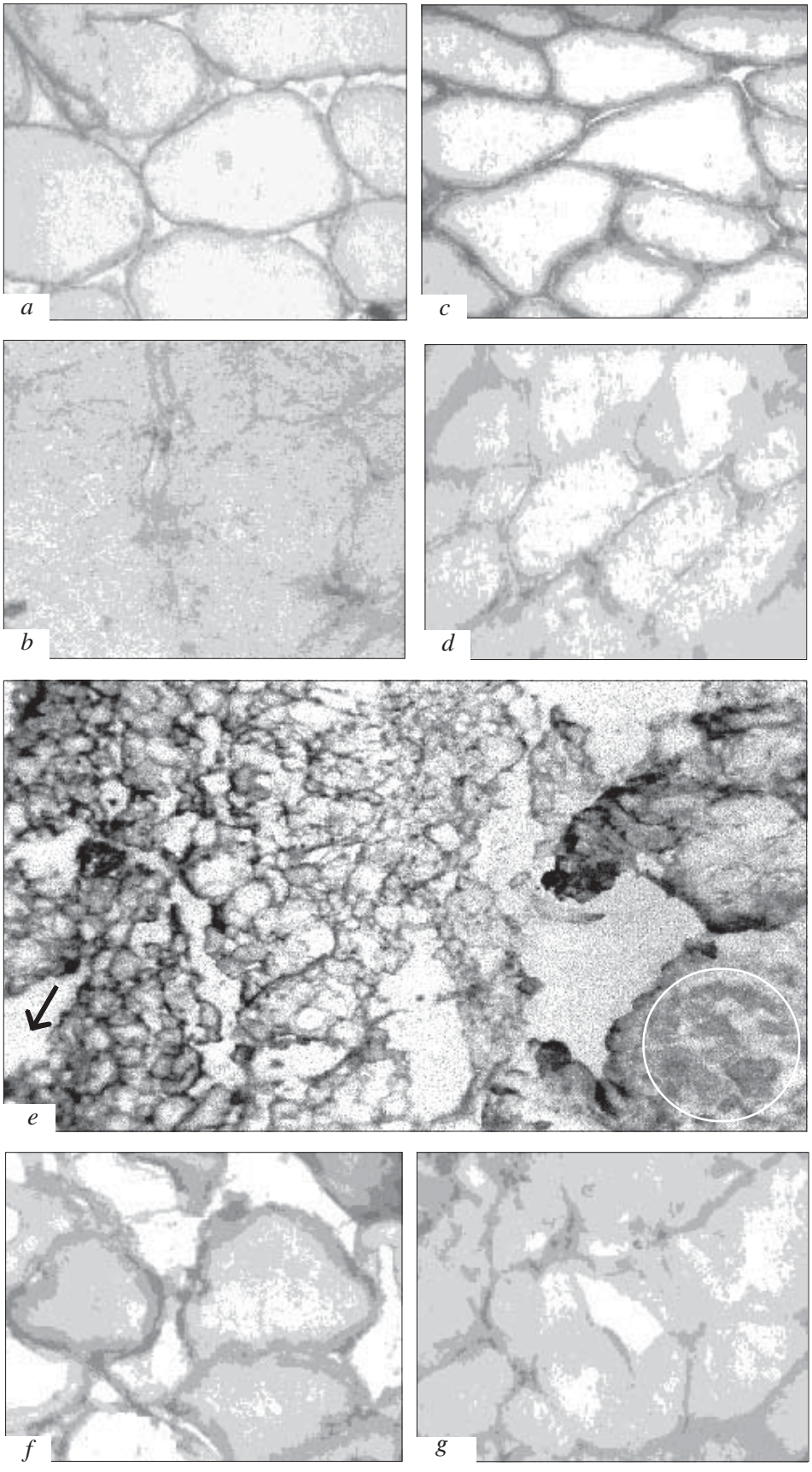

Fig. 3. Immunohistochemical assay for dystrophin. Muscle tissue in a mouse with normal genotype (*a*); muscle tissue in a mouse with mutant genotype (*b*); dystrophin synthesis in muscle tissue of mdx mice 1 month after transplantation (*c*); control muscle (administration of physiological saline, *d*); muscle tissue in a mouse with mutant genotype 3 months after transplantation (arrow: dystrophin-positive fibers; circle: dystrophin-negative fibers; *e*); muscle tissue in a mouse with mutant genotype 5 months after transplantation (*f*); control muscle 5 months after transplantation (*g*).

but found it in normal mice without dystrophin gene mutation. NCL-DYS1 antibodies react with the dystrophin region in exons 26-29. NCL-DYS2 antibodies bind to the CT-region that corresponds to the dystrophin fragment absent in mdx mice. The use of these antibodies allows detection of the synthesis of full-length dystrophin molecule after transplantation of myoblasts. NCL-DYS3 antibodies detect the presence of human dystrophin in the examined tissues. Immunohistochemical assay with antibodies to dystrophin epitopes showed that muscle fibers in the recipient mice infiltrated with human embryonic fibroblasts synthesize dystrophin 1 month after transplantation. In control muscles injected with physiological saline dystrophin synthesis was not detected (Fig. 3, *c*, *d*).

Three months after transplantation some muscle fibers in the recipient mice synthesized dystrophin, while others did not contain this compound (Fig. 3, *d*). Dystrophin characterized by specific near-membrane staining was found in 5-7 muscle fibers of recipient mice 5 months after transplantation of human embryonic myoblasts (Fig. 3, *f*, *g*). In control muscles dystrophin was absent.

Staining with NCL-DYS3 antibodies showed that transplantation of human embryonic myoblasts induced the formation of muscle fibers synthesizing human dystrophin in tissues of mdx mice. Dystrophin synthesis proceeded 1, 3, and 5 months after transplantation without immunosuppression.

Our results show that human embryonic fibroblasts isolated from abortion material at 9-11 weeks' gestation retain viability for a long time after transplantation to recipient muscle. Donor myoblasts migrate along muscle fibers, interact with them, and restore dystrophin synthesis disturbed due to mutation. It should be emphasized that they initiate the synthesis of human dystrophin. We observed no morphological and immunological signs of transplant rejection, which is confirmed by long-term survival of these cells with progressive decrease in their activity and the number of dystrophin-positive fibers. These data indicate that embryonic myoblasts retain low immunogenicity typical of stem cells. This conclusion is derived from the xenogeneic type of transplantation. Our findings indicate that the embryonic tissue from mammals most close to humans holds much promise for the therapy of Duchenne's muscular dystrophy and other hereditary muscle diseases. Further investigations are required to estimate the effects of transplantation of embryonic myogenic precursors from animals to humans. It is necessary to determine the degree and type of functional compensation in genetically deficient muscles after transplantation. Moreover, the risk of complications should be evaluated. These data would provide indications for cell therapy with regard to the age, severity of damage to skeletal muscles, and period of treatment.

REFERENCES

1. G. T. Sukhikh, V. V. Malaitseva, I. M. Bogdanova, *et al*., *Byull. Eksp. Biol. Med.*, **132**, No. 12, 604-612 (2001).
2. V. Brussee, F. Tardif, B. Roy, *et al*., *Transplantation*, **67**, No. 12, 1618-1622 (1999).
3. M. Chen, H. J. Li, Q. Fang, *et al*., *Cell Transplant*., **1**, No. 1, 17-22 (1992).
4. M. A. Goodell, M. Rosenzwelg, H. Kin, *et al*., *Nat. Med*., **3**, No. 12, 1337-1345 (1997).
5. E. Gussoni, H. M. Blau, and L. M. Kunkel, *Ibid*., **3**, No. 9, 970-977 (1997).
6. J. Huard, G. Tremblay, S. Verreault, *et al*., *Cell Transplant*., **2**, No. 2, 113-118 (1993).
7. K. A. Jackson, T. Mi, and M. A. Goodell, *Proc. Natl. Acad. Sci. USA*, **96**, No. 25, 14,482-14,486 (1999).
8. I. Kinoshita, J. T. Vilquin, B. Guerette, *et al*., *Transplant. Proc*., **26**, No. 6, 3518 (1994).
9. P. K. Law, *Lancet*, **341**, No. 8839, 247 (1993).
10. J. R. Mendell, J. T. Kissel, A. A. Amato, *et al*., *N. Engl. J. Med*., **333**, No. 13, 832-838 (1995).
11. C. N. Pagel, J. E. Morgan, J. G. Gross, and T. A. Partridge, *Cell Transplant*., **9**, No. 4, 531-538 (2000).
12. G. M. Smythe and S. I. Hodgetts, *Mol. Ther*., **1**, 304-313 (2000).
13. Y. Torrente, J. P. Tremblay, F. Pisati, *et al*., *J. Cell Biol*., **152**, No. 2, 335-348.
14. J. P. Tremblay, F. Malouin, R. Roy, *et al*., *Cell Transplant*., **2**, No. 2, 99-112 (1993).
15. J. D. White, J. J. Bower, J. B. Kurek, and L. Astin, *Muscle Nerve*, **24**, No. 5, 695-697 (2001).