MORPHOLOGY AND PATHOMORPHOLOGY

Restoration of Gastrocnemius Muscle in MDX Mice of Different Age after Injury and Implantation of Xenogenic Muscle Tissue

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The intensity of regeneration of crossed gastrocnemius muscle was evaluated in two groups of mdx mice of different age 2 weeks after implantation of crushed muscle tissue from newborn rats into the wound defect area. The effect of xenoplasty manifested in increased weight of the damaged muscle. The effect was observed in mice aging 12-16 weeks but not in those aged 40-48-weeks. Structural changes in the skeletal muscle tissue intrinsic of mdx mice and augmenting with age were detected in intact mice before the experiment. Activity of muscle fiber regeneration in intact and injured muscle of 40-48-week-old mice was significantly lower than in 12-16-week-old ones. Myoblasts of the xenogenic transplant retained viability in recipient muscles for at least 2 weeks. Post-traumatic regeneration was stimulated in only 12-16-week animals. Xenoplasty was ineffective in older animals and even somewhat enhanced the destructive processes in the muscle. It seems that age-specific regeneration activity of the recipient skeletal muscle tissue should be taken into consideration in the development of effective strategy of cell therapy for progressive muscular dystrophy.

Key Words: *regeneration; age; cell technologies; tissue therapy; mdx mice*

Manifestations of hereditary muscular dystrophy in mdx mice are largely homologous to Duchenne progressing muscular dystrophy (PMD) in humans. In both cases dystrophin (protein) is absent in muscle fiber sarcolemma because of gene mutation in X-chromosome. Fibers lacking this protein essential for transduction of mechanical signals die during muscle contractions [12]. Partial restoration of dystrophin expression under the effect of myoblasts demonstrated in clinical studies [10,13] seems to be due to "transfection" of normal dystrophin gene into muscle fibers fusing with myoblasts [9].

Muscular dystrophy progresses since birth for about 20 years in boys (potential recipients of myoblasts). Chronic degenerative process in skeletal muscles is paralleled by a decrease in the pool of satellites, forming young muscle fibers replacing dying ones, and decrease in their division capacity [8]. At some moment the degeneration of dystrophin-negative muscle fibers becomes uncompensated and plays the main role in the pathogenesis of skeletal muscles disease. Modern strategies of cell therapies for PMD aimed at re-expression of dystrophin neglect regeneration activity of the reci-

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pient muscle tissue, though it represents the background against which exogenous myoblasts are injected into the muscle. It seems that the effect of therapy is determined by "transfection" of exogenous myoblasts arresting muscle cell necrosis. However, the histogenetic potential of the recipient muscles should not be neglected. We studied the histology of skeletal muscles in mdx mice of different age after injury and therapy with xenogenic myoblasts stimulating histogenesis. Autologous muscle tissue in experiments on genetically healthy animals promotes the filling of the site of injury with muscle fibers regenerating from the autotransplant myoblasts and stimulates the regeneration activity of the recipient damaged muscle fibers [1,2].

MATERIALS AND METHODS

The study was carried out on male mdx mice from the colony maintained at Russian State Medical University. The animals were divided into 2 groups: aged 12-16 and 40-48 weeks. Five mice from each group (subgroup 1) were inflicted the injury, 5 (subgroup 2) received xenogenic tissue implantation after the injury, and 3 intact animals served as controls (subgroup 3). Complete transverse crossing of the gastrocnemius muscle sparing the vessels and innervation of other muscles was carried out in subgroups 1 and 2. Gastrocnemius muscle of 1-2-day-old rat pups was crushed with scissors and implanted to the site of injury to subgroup 2 mice, after which the wound was sutured with silk. The operation was carried out under Nembutal narcosis. After 2 weeks (the period after which the dynamics of the regeneration processes in damaged gastrocnemius muscle can be evaluated at histological examination) the muscles were fixed in Carnoy fluid. Histological sections (7-8 μ) were stained with iron hematoxylin after Regaud with poststaining after Mallory and the weight of muscle regenerate was evaluated in percent of mouse body weight.

RESULTS

The dynamics of changes in the weight of the gastrocnemius muscle in mdx males was evaluated and the efficiency of recovery of this parameter after transplantation of newborn muscle tissue to recipients of different age was compared (Fig. 1). The mean weight of the gastrocnemius muscle in mdx mice aging 40-48 weeks was significantly lower ($p<0.01$). The injury inflicted to 12-16-week animals caused a significant reduction in muscle weight after 2 weeks in comparison with that of intact muscle ($p<0.01$), while in 40-48-week-old mice this parameter only tended to decrease.

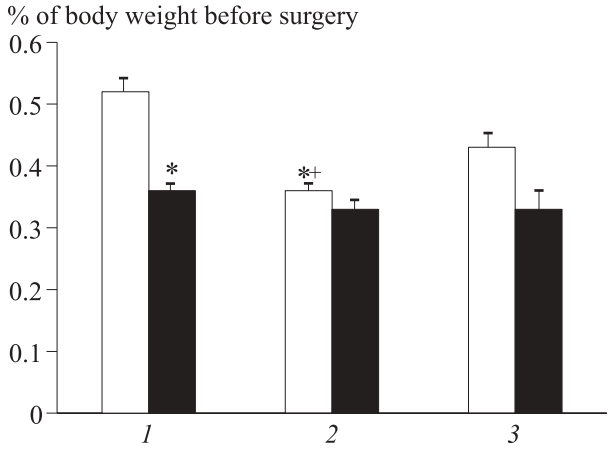

Fig. 1. Gastrocnemius muscle weight 2 weeks after the operation and after implantation of xenogenic muscle tissue to mdx mice. *1*) intact muscle; *2*) 2 weeks after trauma; *3*) injury+implantation. Light bars: 12-16-week-old; dark bars: 40-48-week-old animals. $p<0.01$ compared to *intact muscle in 12-16-week-old mice, +compared to injury+implantation.

The effect of xenotransplantation (a significant increase in muscle weight compared to intact animals, $p<0.01$) was observed only in 12-16-week-old animals.

Significant differences in muscle tissue structure in intact animals of two age groups were detected. The muscle fibers were loose and cleaved, myotubules with a chain of nuclei appeared, dark-colored muscle fibers and fibers with Zenker necrosis were detected in animals aged 12-16 weeks. Destructive processes in muscle tissue progressed in animals aged 40-48 weeks. Abnormal location and lysis of myofibrils were noted. The groups of muscle fibers disintegrate to fragments, which had no cross-striation. The location of the nuclei in muscle fibers was central axial and the fibers differed significantly by the diameter. Accumulation of the connective tissue, adipocytes, and leukocytes in the interstitium was seen. These results attest to decreased activity of spontaneous regeneration with age and agree with published data [11].

Some reports indicate that proliferative activity of myoblasts in mdx mouse and the regeneration capacity of the muscle tissue after injury are preserved [4,5,7]. In our experiments with complete crossing of the muscle the defect area in 12-16-week-old animals was filled with the connective tissue containing fine collagen fibers and proliferating fibroblasts (Fig. 2, *a*). Foci of moderate leukocytic infiltration were detected in the connective tissue. Round muscle nuclei accumulated at the ends of crossed muscle fibers, myotubules and myosymplasts with numerous oval clear nuclei and

large nucleoli formed (Fig. 2, *b*). Muscle fibers with a chain of centrally located muscle nuclei predominated in muscle tissue distant from the site of injury in both stumps. Muscle fibers with Zenker necrosis were more incident than in intact mice.

Regeneration activity of muscle fibers in traumatized 40-48-week-old mice was much lower.

After transplantation of newborn muscle tissue containing up to 40% satellite cells [6] to young mdx mice, activity of regeneratory process in the damaged muscle and activity of regenerative reconstruction of the implant (resorption of tissue degradation products, release and division of myoblasts) varied negligibly in different animals. However, on

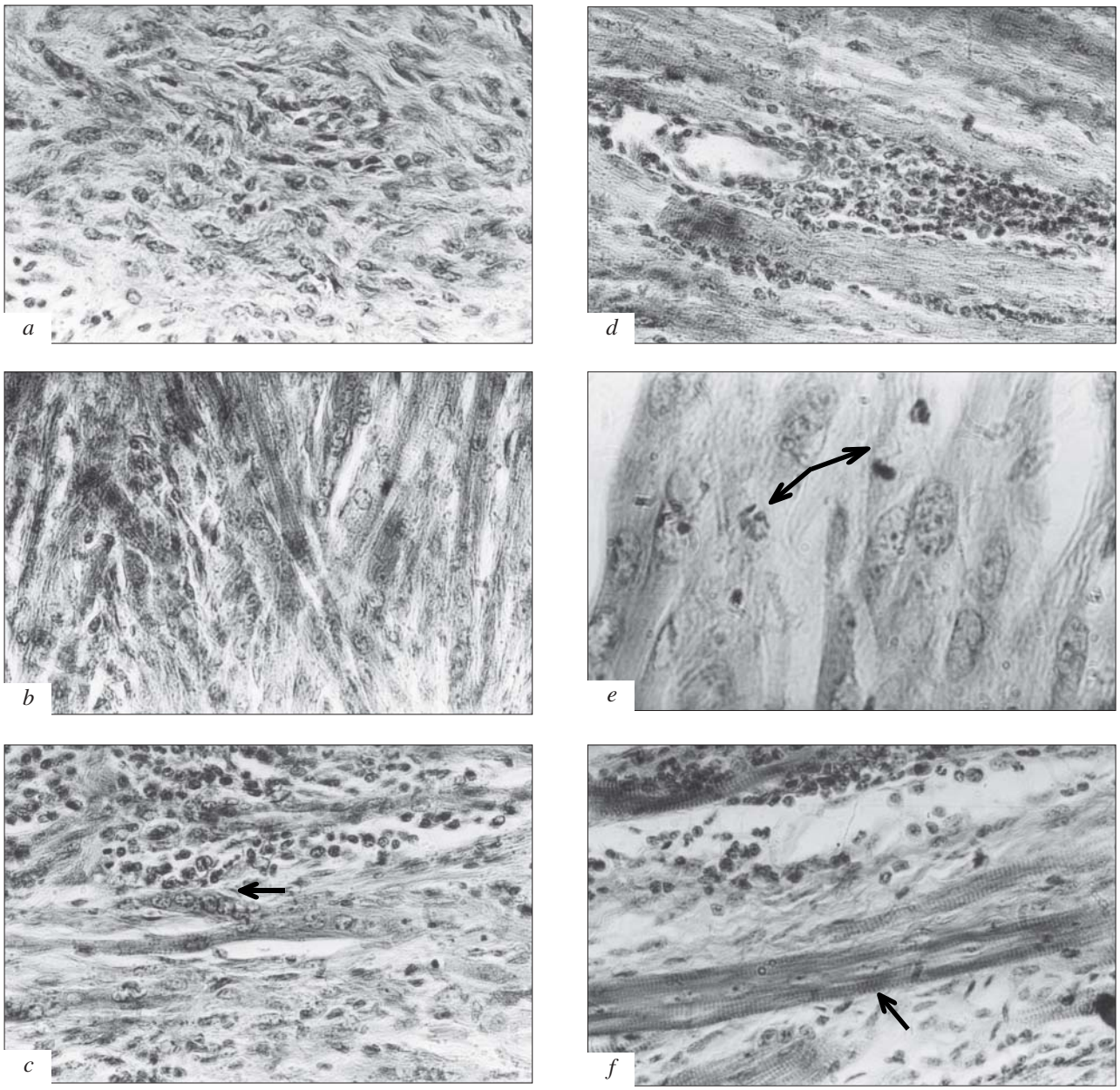

Fig. 2. Gastrocnemius muscle tissue in mdx mice of different age after trauma without implantation (*a*, *b*) and with implantation (*c-f*) of xenogenic tissue 2 weeks after surgery. *a*) connective tissue forming at the site of injury after complete transection of the muscle in 12-16-week-old mice (×400); *b*) muscle fibers regenerating along the edge of the stump after complete ttransection of the muscle in 12-16-week-old mice (×400); *c*) myotubules growing into muscle tissue xenoimplant (arrows) after complete transection of the muscle in 12-16-week-old mice (×400); *d*) muscle fibers growing towards the implant, increased leukocytic infiltration after complete transection of the muscle in 12-16-week-old mice (×400); *e*) myoblasts dividing by mitosis (arrows) near regenerating myotubules at the implant periphery after complete transverse crossing of the muscle in 12-16-week-old mice (×900); *f*) thinning, cleavage of muscle fibers, muscle fibers with central axial position (arrows), increased volume of connective tissue, leukocytic infiltration after complete transection of the muscle in 40-48-week-old mice (×400).

the whole, after 2 weeks myotubules and myosymplasts with few clear nuclei appeared at the implant periphery (Fig. 2, *c*). Muscle fibers from the stumps of the crossed gastrocnemius muscle actively grew into the implant (Fig. 2, *d*).

Mitotically dividing mononuclear cells were often seen in the immediate vicinity of regenerating muscle elements (Fig. 2, *e*). Presumably, this was a result of fusion of viable myoblasts of implanted tissue with regenerating muscle fibers of mutant mice leading to the formation of hybrid muscle fibers. The possibility of fusion of newborn mouse satellite cells with regenerating muscle fibers was shown on adult 129/ReJ, C57Bl/6J, and CBA mice [14].

Muscle fibers were packed very compactly in the muscle sites distant from the injury; their diameter was normal. Dying muscle fibers were detected. Adipocytes appeared in the endomysium. The leukocytes infiltrated mainly the implant and edges of both stumps (Fig. 2, *d*).

In 40-48-week-old mice proliferative activity of muscle elements remained low after implantation of xenogenic muscle tissue. Oval clear muscle nuclei were located alone or by pairs. Solitary muscle fibers grew towards the defect. The implant was gradually resorbed. Macrophages, empty sarcolemma tubes, myoblasts and fibroblasts were detected at the site of xenoplasty.

Xenoimplantation of muscle tissue stimulated destructive processes in the muscle. Xenoimplantation of muscle tissue significantly stimulated the destructive processes in the muscle. Cross striation disappeared at a great distance from the site of injury, sometimes reaching the septae; round nuclei occupied central axial position, but formed no chains and did not accumulate at the terminals (Fig. 2, *f*). Sclerotization increased, the septae were extended and condensed, the number of dark-colored muscle fibers with abnormal location of myofibrils and Zenker necrosis increased, and cleaving muscle fibers were seen in muscle tissue in the entire regenerate. Degeneration of muscle fiber groups into fragments with disappearance of cross striation was observed. In contrast to younger mice, intensive leukocytic infiltration in older animals was seen not only in the xenotransplantation region, but also in the muscle tissue of the proximal and distal parts of the recipient muscle. Leukocytes formed "muffs" around large blood vessels.

According to modern concepts of PMD pathogenesis, re-expression of dystrophin and restoration of skeletal muscle tissue histogenesis in patients are equally important for clinical efficiency of cell therapy. We did not study dystrophin re-expression, but evaluated the capacity of cell material to stimulate histogenesis in damaged muscles of recipients of different age. Xenogenic myoblasts stimulated regeneration activity in muscles of 12-16-week-old mice, but not in older animals. Hence, clinical trials should be carried out after preliminary screening of patients in whom histogenesis in the skeletal muscles cannot be stimulated by myoblasts.

The satellite pool in boys suffering from muscular dystrophy is exhausted after the age of 6 years [9]. More precise evaluation of this threshold will give a check-point for separation of the group of patients potentially "unfit" for myoblast therapy. Analysis of muscle biopsy specimens in these patients will help to evaluate the pool of satellites and their proliferative activity, and analyze the probability of using other cell material for the treatment of PMD.

REFERENCES

1. N. V. Bulyakova, M. F. Popova, S. M. Zubkova, *et al.*, *Laser and Tissue Therapy* [in Russian], Moscow (1993).
2. M. F. Popova, *Radiosensitivity and Stimulating Properties of Mammalian Regenerating Tissues* [in Russian], Moscow (1984).
3. A. N. Studitskii and Z. P. Ignatyeva, *Muscle Repair in Higher Mammals* [in Russian], Moscow (1961).
4. J. E. Anderson and W. K. Ovalle, *Anat. Res.*, **219**, No. 3, 243-257 (1987).
5. D. L. Bourke, M. Ontell, and F. Taylor, *Am. J. Anat.*, **181**, 1-11 (1988).
6. A. E. Hellmuth and D. B. Allbrook, *J. Anat.*, **110**, No. 3, 503 (1971).
7. T. Kagawa, E. Chikata, J. Tani, *et al.*, *Develop. Biol.*, **65**, No. 2, 526-530 (1978).
8. S. S. Jejuricar and W. M. Kuzon Jr., *Apoptosis*, **8**, No. 6, 573-578 (2003).
9. I. Kinoshita, J. T. Vilquin, and J. P. Tremblay, *Acta Neuropathol.*, **91**, No. 5, 489-493 (1996).
10. P. K. Law, T. G. Goodwin, Q. Fang, *et al.*, *Acta Paediatr. Jpn.*, **33**, No. 2, 206-215 (1991).
11. M. Ontell, K. C. Feng, K. Klueber, *et al.*, *Anat. Rec.*, **208**, 159-174 (1984).
12. B. J. Petrof, J. B. Shrager, H. H. Stedman, *et al. Proc. Natl. Acad. Sci. USA*, **90**, No. 8, 3710-3714 (1993).
13. S. S. Shishkin, S. M. Terekhov, T. B. Krokhina, *et al. Genetika*, **37**, No. 8, 1104-1111 (2001).
14. D. J. Watt, K. Lambert, J. E. Morgan, *et al.*, *J. Neurol. Sci.*, **57**, 319-331 (1978).