

Analysis of neogenesis in rabbit skeletal muscles after chronic traction

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Summary. After application of a modified Orthofix mini-extraskelatal fixator, the hind limb of New Zealand White rabbits was osteotomized and then slowly lengthened at a rate of 1 mm/day. After a 20 mm gain in length, the net weight and the length of muscular and tendinous portions were measured and histological examination was carried out in triceps surae muscles. Quantitative analysis showed a significant increase in the gained length of the muscular portion (28.05% to 30.65%). Histological studies of these lengthened muscles showed a generalized increase in cellularity with scanty inflammatory cell infiltration near the myotendinous junction. The increased cellularity is due to the presence of muscle precursor cells characterized by large, oval and pale-stained vesicular nuclei and two prominent nucleoli. The nuclei of these precursor cells were larger and more numerous near the myotendinous junction, and gradually changed into a flattened and more condensed form at a distance from the junction. Occasionally, chains of centrally-located nuclei of primitive myoblasts were also visible. It is concluded that traction neogenesis of the skeletal muscle during limb lengthening does exist and occurs mainly near the myotendinous junction.

Key words: Neogenesis, Rabbit, Skeletal muscle, Traction

Introduction

Ilizarov uses a slow, gradual distraction force as a mechanical signal to stimulate tissue growth, even in mature organ systems. His achievements in both experimental and clinical subjects far surpass the traditional treatment methods (Paley, 1988). The ability of adult skeletal muscle fibres to regenerate after injury has been well documented since the 18th century (Grounds, 1991). Later, numerous investigators (Perth et

al., 1966; Hall-Craggs, 1978; Basson and Carlson, 1980; Garrett et al., 1984; Giddins et al., 1985; Jennische, 1986) reported that adult skeletal muscles did have a great capability of regeneration in response to chemical and physical injury.

In recent years, a series of experiments using the Ilizarov technique to lengthen bones (Aronson et al., 1988) and subsequently to study the mineralization sequence during distraction osteogenesis have been conducted (Aronson et al., 1990). Although Ilizarov clarified the influence of stability of fixation and soft tissue preservation on the genesis of bony tissue (Ilizarov, 1989), the biology of traction neogenesis of soft tissues, especially of muscle fibres, has rarely been studied. The present study has been designed to investigate the biology of traction neogenesis of the skeletal muscles during limb lengthening.

Materials and methods

Nine adult New Zealand White rabbits, weighing 2.0 to 2.5 kg, were used. The rabbits were premedicated with 0.5 mg atropine and then anaesthetized by ketamine (50 mg/kg) and rompun [2-(2,6-xylylidino)-5,6-dihydro-4H-1,3-thiazine hydrochloride] at 12 mg/kg. Bilateral modified Orthofix external fixator equipped with lengthening apparatus was transfixed by four transverse K-wires inserted across the tibia under aseptic condition. After osteotomy of the tibial and fibula diaphyses, lengthening of the leg with a speed of 1 mm per day (one step per day) was performed from the 3rd postoperative day and was ended after 20 mm of lengthening. To prevent the postoperative starvation effect of bilateral osteotomy, the opposite hindlimb was used as nonoperative control. At the completion of traction, the animals were anaesthetized with pentobarbital (0.5 ml/kg), laparatomized and then perfused with a fixative containing 2% paraformaldehyde, and 2% glutaraldehyde in 0.067M cacodylate buffer (pH 7.4) via a cannula through the aorta. At the completion of perfusion, the triceps surae, tibialis anterior and tibialis posterior muscles were carefully dissected from their origin and insertion. The net weight of each dissected

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muscle and the length of the muscular and tendinous portions of each specimen were carefully measured. For histological examination, representative cross and longitudinal paraffin sections, stained with haematoxylin and eosin, of each muscle were taken from the midportion of the muscle belly, the myotendinous junction and the tendon. The cellularity of myotendinous junction of control and experimental rabbit was determined by measuring the mean number of myonuclei in four randomly-selected high-power fields (0.17 mm^2). All the quantitative data were analyzed by paired-t test.

Results

Quantitative analysis

Table 1 shows the quantitative data on the net weight

as well as mean length of the muscular and tendinous portions of each muscle after fixation. There was no significant difference in the net weight of the muscles between control and experimental groups. The difference in the mean length of the muscles (including belly and tendons) was significant between the control and experimentally-lengthened limbs ($P < 0.001$). If we examined the length of belly and tendon separately, a significant increase in the muscular portion but not in the tendinous portion was detected (Table 1).

Histological studies

Gross morphology showed that the lengthened muscles from the experimental group were more slender and longer than the corresponding muscles of the control groups (Fig. 1). Histological examination revealed a striking appearance, i.e., aggregations of nuclei, an

Table 1. Measurements of net weight and length of muscular and tendinous portions of triceps surae, tibialis anterior and tibialis posterior muscles from control and experimentally-tractured animals; (n= 9). All the values were obtained after aldehyde fixation and are expressed as mean±standard deviation.

MUSCLE	CONTROL	EXPERIMENTAL	NET INCREASE	% INCREASE	P VALUE
<i>Net weight (gr)</i>					
Triceps surae	16.7±2.5	15.8±3.5	-0.95±0.14	-5.69±0.84	>0.05
Tibialis ant.	2.6±0.5	2.7±0.9	0.06±0.01	2.28±0.29	>0.05
Tibialis post.	3.3±0.6	3.4±0.9	0.11±0.02	3.35±0.97	>0.05
<i>Length (mm)</i>					
Triceps surae	95.6±4.6	115.0±5.8	19.4±0.90	20.3±0.98	<0.001
Tibialis ant.	109.4±4.6	130.7±4.9	21.3±0.90	19.5±1.87	<0.001
Tibialis post.	85.1±6.2	104.6±6.7	19.5±1.40	22.9±1.67	<0.001
<i>Muscular portion</i>					
Triceps surae	68.1±5.2	87.2±6.3	19.1±1.45	28.05±2.13	<0.0001
Tibialis ant.	65.9±5.8	86.1±5.5	20.2±1.78	30.65±2.70	<0.0001
Tibialis post.	64.3±6.4	83.1±7.6	18.8±1.87	29.23±2.29	<0.0001
<i>Tendinous portion</i>					
Triceps surae	27.1±4.0	27.8±4.4	0.70±0.10	2.58±0.38	>0.05
Tibialis ant.	43.5±5.6	44.6±4.3	1.10±0.14	2.47±0.32	>0.05
Tibialis post.	20.8±6.0	21.5±6.2	0.70±0.20	3.37±0.97	>0.05

Fig. 1. Photomacrograph of triceps surae (TS), tibialis anterior (TA), and tibialis posterior (TP) from the control group (right panel) and experimentally-lengthened limb (left panel). Note the apparent difference in size and length between the corresponding muscles of these two groups.

Fig. 2. Photomicrograph of myotendinous (MT) junction from an obliquely sectioned triceps surae muscle of a control limb. Note the uniform width of the fibre, rather scanty cellularity near the myotendinous junction and the dark, peripherally-sited nuclei of the muscle fibre. T: tendon portion; M: muscle fibre portion. H&E stain. x 250

Fig. 3. Photomicrograph of a longitudinal section of triceps surae muscle from experimental group. Marked increase in cellularity and basophilia of the tissue near the myotendinous (MT) junction are seen. Inflammatory cell infiltration is not obvious in this field. H&E stain. x 250

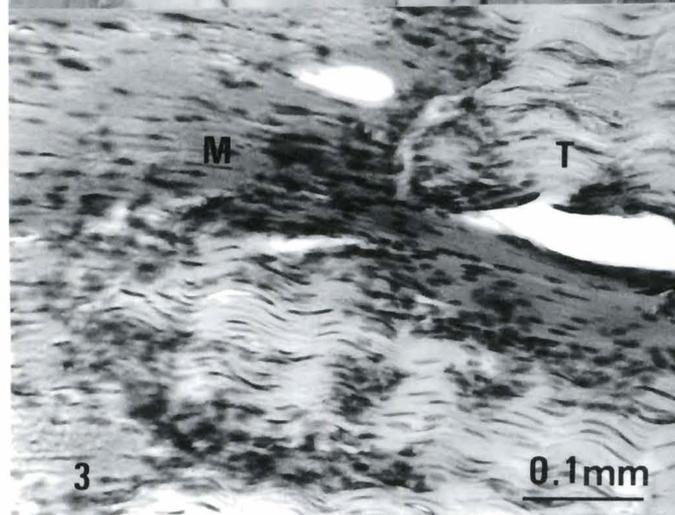
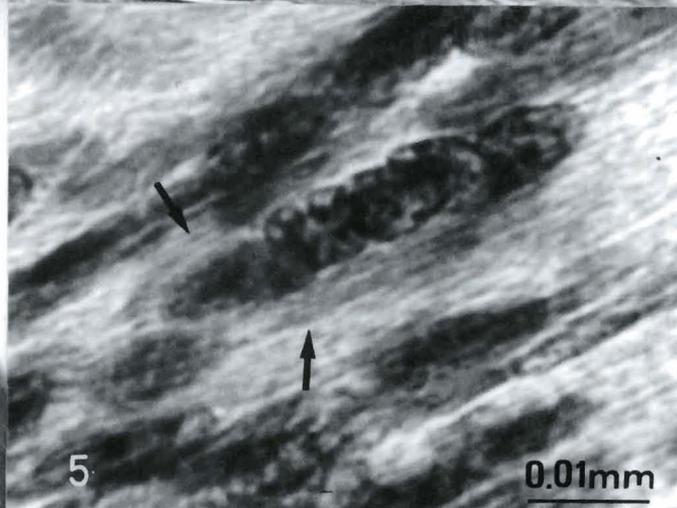
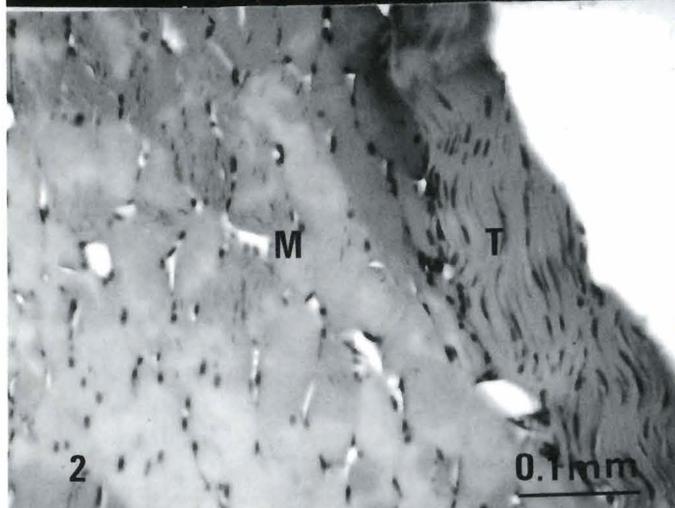
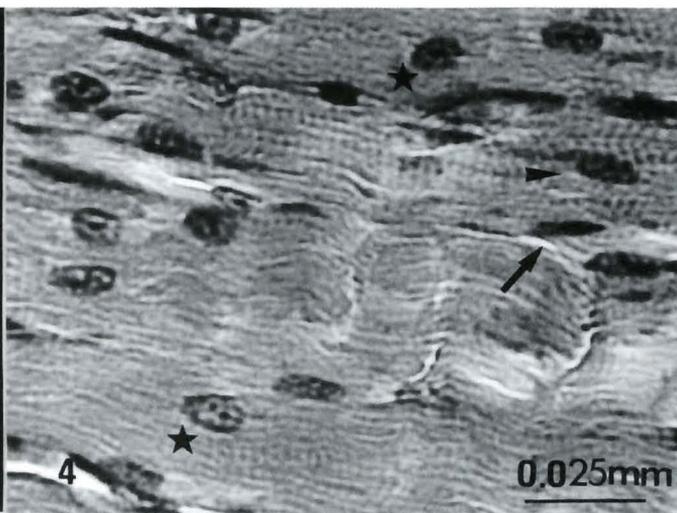
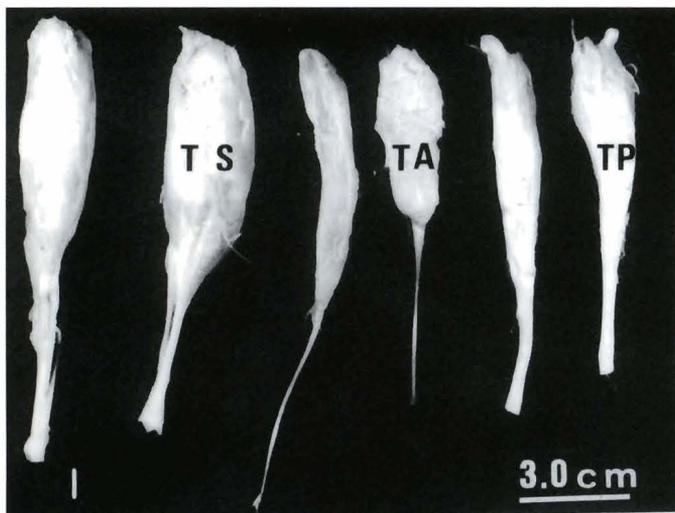
Fig. 4. Higher magnification of myotendinous junction (MT) from longitudinal section of triceps surae muscle from the experimental group. Numerous large ovoid nuclei of myosatellite precursor cells with two prominent nucleoli are visible (*). Note that the nuclei of these myosatellite precursor cells are found not only peripherally (arrow) but also centrally located (arrowheads). H&E stain. x 1,000

Fig. 5. Longitudinal section of triceps surae muscle of the experimental group. Chains of vesicular nuclei of myoblasts can be identified: most of these nuclei have two prominent nucleoli. Newly formed contractile filaments (arrows) can be seen on either side of the myoblast chain. H&E stain. x 2,500

Fig. 6. Cross section of triceps surae muscle from the experimental group at myotendinous junction. In this micrograph, neogenetic fibres are small and variable in size. In their cytoplasm, inter-myofibrillar networks (arrow), are loose, and many nuclei are centrally placed. H&E stain. x 2,500

indication of cellularity and regeneration were found in the myotendinous junction in the lengthened muscles of experimental limbs, but not in control limbs (Figs. 2, 3).

Higher magnification of myotendinous junction revealed that aggregations of nuclei were myoblast syncytia with chains of large, oval and pale-stained vesicular nuclei



and that the majority of these nuclei contained two prominent nucleoli (Fig. 4). Chains of precursor cell nuclei were also visible within this field (Fig. 5). Cross section of the tissue near the myotendinous junction showed variable fibre size and occasional centrally-located nuclei, as well as multiple large nuclei (Fig. 6). As the distance from the myotendinous junction increased, the density of the nuclei decreased gradually and the shape also gradually changed into the normal flat ovoid appearance of mature monocytes. The cellularity of myotendinous junction was 117.8 ± 19.5 cells/ 0.17 mm^2 for control limb and 284.5 ± 38.5 cells/ 0.17 mm^2 . The difference was statistically significant.

Discussion

The extent of regeneration of muscle varies with the nature of injury, but in all situations the process involves necrosis of mature skeletal muscle fibres, cellular infiltration and phagocytosis of damaged muscle, revascularization, proliferation of muscle precursor cells, differentiation and fusion, and finally re-innervation (Grounds, 1991). In the present study, we used a slow, gradual distraction force as a mechanical signal to stimulate the growth of mature skeletal muscle fibres. As shown in Table 1, it is clear that there was an increase in length (19.5% to 22.9%) of whole muscle. The principal lengthening occurred at the muscular portion. The percent of muscle lengthening was 28.05% to 30.65% in this study.

Histological examination in the present study showed that there was a marked increase of the myogenic precursor cells near myotendinous junction, with a gradual maturation of these precursors away from the junctional region. In contrast to previous studies (Perth et al., 1966; Basson and Carlson, 1980; Gutiérrez et al., 1986; Stauber et al., 1990), there were scanty degenerative changes and inflammatory cell infiltration in our series. We speculated that the increase in muscular length mainly originated from neogenesis and not the degenerative-regenerative processes described previously.

Garrett et al. (1987, 1988) and others (Giddings et al., 1985; Almekinders and Gilbert, 1986) demonstrated that whole muscles loaded to failure frequently failed at or near the myotendinous junction. Tidball and Chan (1989) reported that the adhesive strength of muscle cell to basement membrane focused at myotendinous junctions, so the site and stress at failure were independent of strain and strain rate over a biologically relevant range and the failure occurred at myotendinous junctions unless the muscle had suffered previous compression injury to failure within the muscle (Tidball and Chan, 1989). In our present study, mature healthy rabbits were used and there was no evidence of previous muscular injuries. We can expect that the tension effect of prolonged, slow rate of traction (as done in this experiment) would focus mainly upon the myotendinous junction. It has been reported that tension can stimulate

muscle growth *in vitro* (Vandenburg, 1987); therefore, tension is an important regulator of skeletal muscle hypertrophy *in vivo* and *in vitro*. From this point of view, we tend to speculate that the regenerating power of the experimental limbs in our series are due to mechanical stimulation during chronic traction.

Skeletal muscle is capable of regeneration after a second injury, and the rate of this regeneration was even much faster than that of the primary injury (Gulati, 1986). Although the mechanism of this effect is not known; it is suggested that the increased rate and recovery might be due to a conditioning effect of the first injury. Recently, Vandenburg et al. (1990) demonstrated that intermittent repetitive mechanical stimulation could stimulate the growth of differentiated avian skeletal muscle cells *in vitro*. In their experiment, during the first 2-3 hours of stimulation, temporary mechanical damage in the skeletal muscle occurred; however, with continued mechanical stimulation for several days, the protein degradation decreased and stretch induced cell growth. Accordingly, we suggest that a chronic, slow rate traction of the skeletal muscle (1 mm/day in this experiment) can induce cell growth without significant damage to individual cells as evidenced by minimal inflammatory cell infiltration.

Gulati (1988) claimed that the presence of intact innervation was crucial for the terminal differentiation and maturation of regenerating muscles. Denervation resulted in progressive atrophy of muscle, marked by a reduction in the size of myofibres, an increase in endomysial and/or perimysial connective tissue and only a partial maturation of myotubes (Gulati, 1988). In our study, nerve function was well preserved, as evidenced by intact motor function at the end of the experiment; and therefore, we were not surprised to find gradual maturation within the neogenetic muscles without any significant atrophy in the operated muscles (Table 1).

In a study on the regeneration capability at the myotendinous junction, Baker and Poindexter (1991) found that destruction of segmental fibres followed by removal by macrophages occurred at the ends of the soleus muscle and that this destruction resulted in the liberation and myogenic activation of satellite cells. They further reported that three days after tenotomy, myoblasts fused to form myotubes, which developed within the original basal lamina, reattached to the surviving non-necrotic segments and grew both in length and width, and that by 6 weeks postoperatively, normal myotendinous junctions had been reformed. In our model, a slow, chronic intermittent traction developed in the whole muscle. An empty space observed between tendon and muscle cell may be an artifact or a mechanical damage induced by traction during the experiment (Fig. 4). As noted above, we would expect that the stress would be focused upon the myotendinous junction, where numerous myosatellite precursor cells existed. Therefore, muscle cell growth near the myotendinous junction might be induced by the chronic repetitive stretch exerted by traction in the present study.

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A study on the detailed morphological changes on traction neogenesis by electron microscopy and immunocytochemistry is in progress.

In summary, we conclude that traction neogenesis of the skeletal muscle during limb lengthening mainly develops in the area of myotendinous junction. Further studies are required to clarify these biological mechanisms and factors influencing traction neogenesis at cellular and molecular basis.

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References

- Almekinders L.C. and Gilbert J.A. (1986). Healing of experimental muscle strains and the effects of nonsteroidal anti-inflammatory medication. *Am. J. Sports Med.* 14, 303-308.
- Aronson J., Harrison C.M., Boyd B., Cannon D.J., Lubansky H.J. and Stewart C. (1988). Mechanical induction of osteogenesis. Preliminary studies. *Ann. Clin. Lab. Sci.* 18, 195-203.
- Aronson J., Good B., Stewart C., Harrison B. and Harp J. (1990). Preliminary studies of mineralization during distraction osteogenesis. *Clin. Orthop.* 250, 43-49.
- Baker J.H. and Poindexter C.E. (1991). Muscle regeneration following segmental necrosis in tenotomized muscle fibers. *Muscle-Nerve* 14, 348-357.
- Basson M.D. and Carlson B.M. (1980). Myotoxicity of single and repeated injections of mepivacaine (carbocaine) in the rat. *Anesth. Analg.* 59, 275-282.
- Garrett W.E. Jr., Seaber A.V., Boswick J., Urbaniak J.R. and Goldner J.L. (1984). Recovery of skeletal muscle after laceration and repair. *J. Hand Surg.* 9A, 94, 683-692.
- Garrett W.E., Safrani M.R., Seaber A.V., Glisson R.R. and Ribbeck B.M. (1987). Biomechanical comparison of stimulated and nonstimulated muscle pulled to failure. *Am. J. Sports Med.* 15, 448-454.
- Garrett W.E., Nikolaou P.K., Ribbeck B.M., Glisson R.R. and Seaber A.V. (1988). The effect of muscle architecture on the biomechanical failure properties of skeletal muscle under passive extension. *Am. J. Sports Med.* 16, 7-12.
- Giddings C.J., Neaves W.B. and Gonyea W.J. (1985). Muscle fiber necrosis and regeneration induced by prolonged weight-lifting exercise in the cat. *Anat. Rec.* 211, 133-141.
- Grounds M.D. (1991). Towards understanding skeletal muscle regeneration. *Pathol. Res. Pract.* 187, 1-22.
- Gulati A.K. (1986). Pattern of skeletal muscle regeneration after reautotransplantation of regenerated muscle. *J. Embryol. Exp. Morphol.* 92, 1-10.
- Gulati A.K. (1988). Long-term retention of regenerative capability after denervation of skeletal muscle, and dependency of late differentiation on innervation. *Anat. Rec.* 220, 429-434.
- Gutierrez J.M., Chaves F., Mata E. and Cerdas L. (1986). Skeletal muscle regeneration after myonecrosis induced by *Bothrops asper* (terciopelo) venom. *Toxicon* 24, 223-231.
- Hall-Craggs E.C.B. (1978). Ischemic muscle as a model of regeneration. *Exp. Neurol.* 60, 393-399.
- Ilizarov G.A. (1989). The tension stress effect on the genesis and growth of tissue: Part I. The influence of stability of fixation and soft tissue preservation. *Clin. Orthop.* 238, 249-281.
- Jennische E. (1986). Rapid regeneration in postschaemic skeletal muscle with undisturbed microcirculation. *Acta Physiol. Scand.* 128, 409-414.
- Paley D. (1988). Current techniques of limb lengthening. *J. Pediatr. Orthop.* 8, 73-92.
- Perth D.A., Baker W.C. and Kirkaldy-Willis W.H. (1966). Muscle regeneration in experimental animals and in man: The cycle of tissue change that follows trauma in the injured limb syndrome. *J. Bone Joint Surg.* 48B, 153-169.
- Stauber W.T., Fritz V.K. and Dahlmann B. (1990). Extracellular matrix changes following blunt trauma to rat skeletal muscles. *Exp. Mol. Pathol.* 52, 69-86.
- Tidball J.G. and Chan M. (1989). Adhesive strength of single muscle cells to basement membrane at myotendinous junctions. *J. Appl. Physiol.* 67, 1063-1069.
- Vandenburg H.H. (1987). Motion into mass: how does tension stimulate muscle growth? *Med. Sci. Sports Exerc.* 19 (Suppl), S142-S149.
- Vandenburg H.H., Hatfaludy S., Sohar I. and Shansky J. (1990). Stretch-induced prostaglandins and protein turnover in cultured muscle. *Am. J. Physiol.* 259, C232-C240.

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