Relative ability of young and mature muscles to respond to limb lengthening

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The response of the muscle is critical in determining the functional outcome of limb lengthening. We hypothesised that muscle response would vary with age and therefore studied the response of the muscles during tibial lengthening in ten young and ten mature rabbits. A bromodeoxyuridine technique was used to identify the dividing cells.

The young rabbits demonstrated a significantly greater proliferative response to the distraction stimulus than the mature ones. This was particularly pronounced at the myotendinous junction, but was also evident within the muscle belly.

Younger muscle adapted better to lengthening, suggesting that in patients in whom a large degree of muscle lengthening is required it may be beneficial to carry out this procedure when they are young, in order to achieve the optimal functional result.

The response of striated muscle during limb lengthening is a major determinant of functional outcome. Major complications such as contractures, subluxations or dislocations can result from the failure of muscle-tendon units to attain an adequate length.¹⁻³ Although there have been some accounts of the role of the myotendinous junction in the longitudinal growth of muscles during limb lengthening,^{4,5} there is no information available on its proliferative activity at different ages and rates of distraction, or comparing proliferative activity at the myotendinous junction to the proliferative response within the muscle belly.⁶⁻¹⁰ The aim of this study was to provide detailed information on the proliferative activity of myogenic cells in the myotendinous junction compared with that of the muscle belly in young and mature rabbits in which the tibia had been lengthened at different rates.

Materials and Methods

Tibial osteotomy and stabilisation with an external fixator (ITEC mini distractor MC-SCD-002; ITEC, Budapest, Hungary; Fig. 1) was carried out on 20 male New Zealand White Rabbits. The left hind limb was lengthened in 16 rabbits (eight young and eight mature; of which four young and four mature animals were distracted at a rate of 0.8 mm/day and four young and four mature at a rate of 0.8 mm twice daily. The unoperated right hind limb served as a control in the comparison of muscle length (Table I). Four rabbits underwent osteotomy

and stabilisation, but not limb lengthening (distraction rate 0 mm/day). The young rabbits were nine weeks old and the mature rabbits 28 weeks old,¹¹ at which time longitudinal growth has just stopped, as in a young adult human. All of the rabbits were killed immediately after completion of the lengthening procedure. The experiment conformed to national regulations and Ethics Committee approval was obtained. **Operative technique.** The animals were randomly allocated to the groups. The operation was undertaken using the technique previously described.^{6,11,12} After shaving and draping the left tibia, the skin and periosteum were incised longitudinally under general anaesthesia using intravenous ketamine hydrochloride and diazepam, titrated as appropriate.^{6,12} Four screws were placed along the longitudinal axis of the tibia and the external fixator was positioned. A transverse osteotomy of the tibia was carried out 8 to 15 mm distal to the tibiofibular junction, junction in the middle portion of the diaphysis (Fig. 1).

After seven days, lengthening was started in the experimental groups. The bony ends were distracted in increments of 0.8 mm, once or twice daily, depending on the rate of lengthening. The length of the tibia was increased by 20% in every animal.

In order to label the dividing cells, the animals were injected intravenously with a thymidine analogue, bromodeoxyuridine (BrdU) (Sigma Chemical, Poole, United Kingdom) at a dose of 40 mg/kg (8 mg/ml saline) one hour

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Table I. The mean length (SD) of the flexor digitorum longus muscle in the different groups (in mm)

	Young rabbits		Mature rabbits		
	0.8 mm/day	0.8 mm twice daily	0.8 mm/day	0.8 mm twice daily	0 mm/day
Mean length					
Experimental side	76.87 (2.78)	77.6 (4.63)	84.6 (8.15)	84.7 (5.6)	72.6 (12.4)
Control side	68.17 (1.59)	68.4 (3.58)	76.9 (6.07)	74.4 (5.3)	72.3 (12.2)
Length increase (%)	12.7	13.4	10	13.8	0.4

Fig. 1

Radiograph of the lengthened tibia and the external fixator.

before they were killed. BrdU competes with thymidine for incorporation into DNA.¹³ After incorporation into the DNA, the BrdU is stable.

Once lengthening of 20% had been achieved, the animals were killed by an overdose of intravenous barbiturate. The muscles and their tendons were carefully dissected from the bone and spread out on swabs soaked with saline at body temperature. The length of the flexor digitorum longus (FDL) muscle was measured from the proximal end of the intramuscular tendon plate to the distal end of the myotendinous junction, using a Vernier calliper (Sealey, Bury St Edmunds, United Kingdom). The muscles and tendons were processed as previously described.^{6,11,12} Tissues were fixed in 10% phosphate-buffered formalin for 48 hours. After fixation the samples were embedded in paraffin and 5 µm longitudinal sections were cut parallel to the muscle fibres.

Fig. 2

Bromodeoxyuridine-labelled positive staining satellite cell in the muscle near the myotendinous junction (x 400).

Fig. 3

Satellite cell near the myotendinous junction (polarised; x 400).

Monoclonal antibodies (Bu30a) (Sigma Chemical) highly specific to BrdU were used to detect its presence in the cell nuclei, as described by Magaud et al.¹⁴ For counterstaining of the negative nuclei, a weak Mayer's haematoxylin stain was used which was sufficiently strong to demonstrate the negative nuclei but not strong enough to mask weak positive reactions (Figs 2 and 3). Two serial sections of small bowel served as positive controls and two serial sections of

Fig. 4

The positive staining index in the muscle belly of distracted and distracted at 0 mm/day muscles (mean and 95% confidence interval).

Fig. 5

The positive staining index at the myotendinous junction of distracted and distracted at 0 mm/day muscles (mean and 95% confidence interval).

Table II. The mean positive staining index (%) in the muscle belly and in the myotendinous junction in the different groups. The p-value is calculated for the difference between young and mature rabbits

	Young (SD)	Mature (SD)	p-value
Musculotendinous junction			
0 mm/day	8.6 (3.19)	1.92 (0.41)	< 0.005
0.8 mm/day	14 (4.92)	4.67 (1.87)	< 0.007
0.8 mm twice daily	28.4 (10.3)	8.6 (3.07)	< 0.02
Belly			
0 mm/day	2.7 (0.97)	0.34 (0.16)	< 0.003
0.8 mm/day	4.7 (1.6)	1.67 (0.47)	< 0.01
0.8 mm twice daily	12 (4.64)	4.9 (1.63)	< 0.03

liver as negative controls in each batch. The BrdU-labelled muscle nuclei were counted under the light microscope with a magnification of $\times 20$, using an eyepiece. The nuclei were counted in 20 fields to determine the mean positive staining index, which is the number of positive fibre nuclei divided by the total number of muscle fibre nuclei examined $\times 100$.^{6,11} The eyepiece was placed and directed along the line of the myotendinous junction to examine a strip 50 μm wide.

The Mann-Whitney U-test was used to determine significant differences between the groups (independent samples) and Wilcoxon's matched-pairs test for the comparison of experimental and control sides (dependent samples). Both of these tests are non-parametric (level of significance $p < 0.05$).

Results

In the group distracted at 0 mm/day there was no significant difference between the length of the FDL muscle on the experimental side and on the control side. The length of the muscle increased significantly in all other groups when the

Fig. 6

The ratio between the positive staining index of the myotendinous junction and the muscle belly. In young rabbits the dominance of the junction is less pronounced in the distracted at 0 mm/day muscle, but it is better preserved during distraction.

experimental side and the control side were compared (Table I).

The positive staining index of all specimens examined is shown in Table II.

In the group distracted at 0 mm/day there was no significant difference between the positive staining index of the experimental and control sides. In all of the lengthened limbs a significant increase of positive staining index in the muscle belly and at the myotendinous junction was observed, compared with the unlengthened control limb ($p < 0.01$). The relative increase in positive staining index in the muscle belly was greater than that observed in the myotendinous junction (Figs 4 to 6).

At both rates of distraction, the response to lengthening in the young was far greater than in the mature animals, being over three times more at the myotendinous junction and over double in the muscle belly. On average, there were nearly six times more dividing myogenic cells in the myotendinous junction than in the muscle belly in the non-distracted muscles of mature rabbits, and a little over three times more in young rabbits (Fig. 6). In mature animals we observed a pronounced shift in the distribution of dividing cells during distraction, as although the positive staining index in the myotendinous junction remained greater than in the muscle belly, the ratio between the two decreased significantly during distraction ($p < 0.02$; Fig. 6). This change was the result of an increased contribution of the muscle belly to proliferation.

Discussion

Moss and LeBlond¹⁵ demonstrated that the satellite cells are responsible for the increase in the number of myonuclei which occurs during post-natal growth. These cells play an integral role in the normal development of skeletal muscle by providing a source of post-mitotic myonuclei. However, the number of satellite cells in a muscle undergoes an ordered progressive decrease over the course of post-natal life,¹⁶ and we observed significantly fewer satellite cells in the mature than in the young animals. Schumacher, Keller and Hvid¹⁷ were the first to use BrdU to label the myonuclei derived from the satellite cells, but they did not examine the myotendinous junction and were therefore unable to compare the behaviour of this site with that of the muscle belly. In order to avoid labelled fibrocytes in the close proximity of muscle fibres being counted erroneously as satellite cells, we used longitudinal sections, which helped us to distinguish these two types of cell. Our results showed an increase in the number of labelled myonuclei in the muscle belly with distraction, as observed by Schumacher et al.¹⁷ We have also demonstrated an increase in the activity in the myotendinous junction. As proliferation of satellite cells result in the formation of myofibrils,¹⁸ although the myotendinous junction is a key site in the longitudinal growth of striated muscles, the previous view that the growth of muscle fibres occurs exclusively at the end of fibres should be revised.^{4,18,19}

Simpson et al⁶ found that limbs of skeletally-mature animals which were lengthened at a rapid rate developed contractures. However, even at rapid rates of distraction, these authors demonstrated that new sarcomeres were added

with the creation of new muscle tissue. Our results support these findings.

It has previously been suggested that muscle contractures associated with distraction osteogenesis may be a function of age.²⁰ We agree with this, and have clearly demonstrated that younger muscle has a better growth potential during limb lengthening. This should be borne in mind when planning programmes where a large amount of lengthening is required.

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