Distraction effects on muscle

Leg lengthening studied in rabbits

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We investigaed changes in the anterior tibial muscle during lengthening of the lower leg in rabbits. In 37 rabbits, an osteotomy of the right middle tibia was performed and was fixed by a unilateral external fixator. The rabbits were randomized into 6 groups. In groups 1, 2, and 3 the tibiae were distracted 0.5 mm/day. In groups 1 and 2, the rabbits were killed after 14 and 28 days of distraction, respectively, and in group 3 after 28 days of distraction, followed by 14 days of rest. Groups 1a, 2a, and 3a served as controls. They were treated similarly as groups 1, 2, and 3, but no distracton was performed. Proliferating cell nuclei were labeled with 5-bromo-2-deoxyuridine and were identified by immunohistochemical staining. The weight of the muscle was measured. During bone lengthening the muscle showed signs of growth, as indicated by increasing weight and number of proliferating cell nuclei. This was observed only during lengthening and it ceased when the lengthening was stopped.

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The amount of limb lengthening is limited by soft tissues rather than bone, muscle being an important factor (Paley 1988, Yasui et al. 1991). Few studies have addressed muscle growth during leg lengthening (Paley 1988, Sproul and Price 1992a, b). Using electron microscopy, Ilizarov (1989a, b) stated that soft tissue subjected to a slow steady pull of tension responds by activating cells. Kawamura (1968, 1981) found that enzyme levels rose during lengthening.

Using DNA-replication as a measurement of proliferating nuclei, we studied the reaction of muscle during leg lengthening in rabbits.

Animals and methods

37 New Zealand white rabbits, 5–6 months old, weighing 3–4.5 kg, were randomized into 6 groups. In all the rabbits, an osteotomy was performed in the middle of the right tibia under Hypnorm/Stesolid intramuscular anesthesia and under antibiotic cover (Ampicillin). A unilateral dynamic external fixation device (Orthofix M-100) was fixed to the medial side of the right leg, with 2 screws above and 2 screws below the osteotomy. Distraction was started after 4 days. The fixator was distracted in a controlled fashion at the rate of 0.5 mm once a day. The rabbits in group 1 (n 6) were distracted for 14 days and then killed. In group 2 (n 7), the rabbits were distracted for 28 days and then killed. In group 3 (n 6), the rabbits were distracted for 28 days and killed after a further 14 days. The rabbits in groups 1a (n 6), 2a (n 6) and 3a (n 6) were operated on similarly with an osteotomy and a fixator, but no distraction was performed, and the groups served as controls. The animals were injected intraperitoneally with bromodeoxyuridine (BrdU) 5 mg/100 g twice, 24 h and 1 h prior to killing. The animals were killed with an intracardiac overdose of barbiturate. The distractor was removed from the right leg. The anterior tibial muscles from both legs were dissected and weighed on a precision scale. Transverse sections from the middle and proximal parts of the muscles were cut for immunohistochemical detection of replicating cells. The dissected tibiae were examined with standardized radiographs having a known magnification in anteroposterior and lateral projections. From the radiographs, the distracted length and the total length of the tibiae (from the tibia condyle to the ankle joint) were calculated.

Staining method

The immunohistochemical technqiues used to detect cells in the process of DNA-synthesis, in this study have been published in detail previously (Langkilde

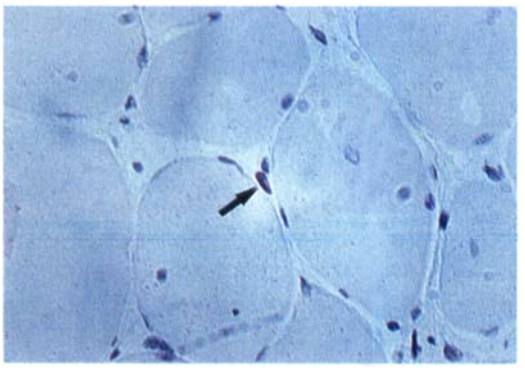

Figure 1. A BrdU-labeled nucleus (arrow) in the distracted anterior tibial muscle. Mayers hematoxylin, ×200.

et al. 1989). The tissue was embedded in paraformaldehyde and cut in 4-μ-thick sections, deparaffinated and rehydrated. The endogenous peroxidase activity was blocked with 2.5 percent H_2O_2 (35 percent) in methanol for 30 min, followed by incubation with pepsin (sigma P-7012) 4 mg/mL in HCL 0.1 N for 60 min. The specimens were DNA-denaturated with HCL 1.0 N for 30 min, incubated overnight with mouse anti-BrdU antibody (Dako A/S Denmark X 902/Becton Dickinson) and detected by means of a biotinylated rabbit anti-mouse Ig (Dako A/S Denmark E 354), followed by an avidin-biotin-complex (Vector Laboratories Inc.). The sections were then incubated with color development, 0.004 percent 3-amino-9-methyl-carbazole (Merck 10983) with 0.01 percent hydrogen peroxide for 10 min, and the reaction was stopped with distilled water. Counterstaining was done in Mayers hematoxylin (Figure 1). Positive and negative reactions were included in order to exclude nonspecific staining. In addition, a section from the jejunum (high turnover of cells) in every rabbit was prepared as a control of the BrdU procedure.

Counting of proliferating nuclei

BrdU-labeled muscle nuclei were counted under the light microscope with a magnification of ×20, using an eyepiece graticule. The nuclei were counted in 20 fields to determine the average BrdU-labeling index, expressed as the number of positive muscle nuclei divided by the total number of muscle nuclei examined × 100. The coefficient of variation was 19 percent.

Statistics

The unpaired Mann-Whitney test was used to compare differences between groups. The distracted group and the control group were compared, using the difference between the right and left legs to minimize the variations between the animals. *P* values less than 0.05 were considered significant.

Results

4 rabbits were excluded from the study: 3 rabbits (groups la, 2 and 3a) suffered from a fracture in one of the drill holes and 1 rabbit (group 3a) died of an unknown cause.

After 14 days of distraction, the tibiae in group 1 were lengthened (median value (25/75 percentiles)) 6.5 (6.0–7.0) mm. The tibiae in group 2 were lengthened 10.5 (9.0–12.8) mm, and the tibiae in group 3 were lengthened 12.0 (11.3–12.8) mm. Lengthenings of the whole tibia were 6 percent, 10 percent and 11 percent in the three groups, espectively. Histological evaluation showed inflammation in and near the fascia in some of the sections from muscle distracted for 14 days. No other signs of inflammation were noted, and edema was not seen in distracted muscle. After 14 days of distraction, no changes in the weight of the tibial muscle or in the number of proliferating cell nuclei were found, compared to the corresponding control group (Tables 1 and 2). After 28 days of distraction, there was a significant increase in the weight of the tibial muscle and in the number of proliferating cell nuclei compared with the corresponding control group. After 28 days of

Table 1. Weight of the tibialis anterior muscle (g). Distraction for 14 days (group 1), distraction for 28 days (group 2) and distraction for 28 days, followed by 14 days rest (group 3). Median values (25–75 percentiles)

	Distracted				Control		
Group	Operated right leg	Nonoperated left leg	Difference (right–left leg)	Group	Operated right leg	Nonoperated left leg	Difference (right–left leg)
1, n 6	8.5 (7.6–8.7)	8.6 (7.4–9.2)	–0.1 (–0.2–0.3)	1a, n 5	6.6 (6.3–7.2)	6.7 (6.2–7.7)	–0.3 (–0.6–0.0)
2, n 6	9.2 (9.0–9.5)	7.6 (7.1–8.1)	1.2[a] (0.9–2.0)	2a, n 6	7.5 (7.4–8.1)	8.0 (7.3–9.3)	–0.6 (–0.8–0.2)
3, n 6	10.1 (9.5–10.4)	8.6 (8.0–9.1)	1.3[a] (1.1–2.0)	3a, n 4	8.0 (7.3–9.8)	8.0 (7.2–9.4)	0.2 (–0.1–0.6)

[a] $P < 0.05$ compared with the control group.

Table 2. Number of labeled cell nuclei in percent of the total number of cell nuclei in proximal and middle sections in the tibialis anterior muscle. Distraction for 14 days (group 1), distraction for 28 days (group 2) and distraction for 28 days, followed by 14 days' rest (group 3). Median values (25–75 percentiles)

Distracted				Control			
Group	Operated right leg	Nonoperated left leg	Difference (right–left leg)	Group	Operated right leg	Nonoperated left leg	Difference (right–left leg)
Proximal sections							
1, n 5	0.1 (0.0–0.2)	0.0 (0.0–0.2)	0.0 (0.0–0.1)	1a, n 5	0.0 (0.0–0.1)	0.1 (0.0–0.4)	0.0 (–0.4–0.0)
2, n 6	2.0 (1.4–2.9)	0.1 (0.1–0.3)	1.9[a] (0.8–2.8)	2a, n 6	0.1 (0.0–0.2)	0.0 (0.0–0.1)	0.0 (0.0–0.2)
3, n 6	0.3 (0.0–0.4)	0.0 (0.0–0.1)	0.2 (0.0–0.3)	3a, n 4	0.0 (0.0–0.0)	0.0 (0.0–0.1)	0.0 (–0.1–0.0)
Middle sections							
1, n 5	0.1 (0.1–0.3)	0.0 (0.0–0.0)	0.1 (0.1–0.3)	1a, n 5	0.0 (0.0–0.4)	0.0 (0.0–0.3)	0.0 (–0.3–0.4)
2, n 6	1.8 (1.1–2.9)	0.1 (0.0–0.3)	1.5[a] (1.1–2.6)	2a, n 6	0.1 (0.1–0.1)	0.0 (0.0–0.0)	0.1 (0.1–0.1)
3, n 6	0.0 (0.0–0.5)	0.0 (0.0–0.2)	0.0 (0.0–0.2)	3a, n 4	0.1 (0.0–0.1)	0.0 (0.0–0.0)	0.1 (0.0–0.1)

[a] $P < 0.05$ compared with the control group.

distraction and 14 days of rest, there was still a significant increase in the weight of the tibial muscle, but the number of proliferating cell nuclei was the same as in the corresponding control group. No significant difference in the number of proliferating cell nuclei was found between sections from the proximal and from the middle parts of the tibial muscle in the group of rabbits distracted for 28 days. In sections from the jejunum, we found numerous stained cells in all rabbits but one, and this rabbit was excluded when labeled nuclei were counted (group 1).

Discussion

Detection of cells in the process of DNA-synthesis (S-phase of the cell cycle) is performed by using 5-bromo-2-deoxyuridine (BrdU). BrdU, a pyrimidine analogue of thymidine, is incorporated in nuclear DNA during DNA-synthesis. Cells replicating in an environment containing BrdU, administrated in vivo or in vitro, are labeled. They can be identified by the use of an anti-BrdU monoclonal antibody (Langkilde et al. 1989). In investigations on rapidly growing tumors, immunohistochemical labeling of proliferating cells using BrdU has proved to be a practical and valid method (Langkilde et al. 1989, Wilson 1991). In our study the frequency of mitosis is low, compared to a malignant tumor. However, by increasing the number of fields counted, we found an acceptable coefficient of variation (19 percent).

Ilizarov (1989a), using electron microscopy, observed that soft tissue subjected to stretch responds by activating the entire cell apparatus (hypertrophy of the Golgi complex, enlargement of the mitochondria). Accordingly, an increase in oxidative enzyme activities was found in the muscle after a one-stage stretching of chicken wing muscle (Holly et al. 1980) and after a rapid one-stage 10 percent leg lengthening in dogs (Kawamura 1968, 1981).

The present study demonstrates signs of growth of the anterior tibial muscle during bone lengthening of approximately 10 percent in rabbits, as indicated by an increase in the weight of the muscle and the number of proliferating cell nuclei. Correspondingly, myogenesis was found after 10 and 20 percent lengthenings by Dyachova (quoted by Paley, 1988). In contrast, after a 20 percent lengthening, Lee et al. (1993) found histopathological changes, such as endomysial fibrosis and internalization of nuclei, suggesting irreversible damage to the muscle.

Normal muscle growth appears to occur primarily in the proximal end of the muscle, at the musculotendinous junction (Williams and Goldspink 1971). After lengthening we found an increase in the muscle cell number both in the proximal and in the middle parts (site of the osteotomy) of the muscle. Our results are in agreement with those of Yasui et al. (1991), who marked the fascia and found that the increase in muscle length occurred throughout the muscle belly.

Satellite cells of the muscle play an integral role in the normal development of skeletal muscle (Schultz 1989). Increased functional demand results in increased satellite cell mitotic activity. Following a mitotic division, the daughter cells fuse with the adjacent myofiber, thereby contributing a small amount of cytoplasm. It is probably the satellite cells that account for the increase in the number of proliferating cells in the distracted muscle. Holly et al.

(1980) reported muscle hypertrophy of the muscle fiber caused by stretching the muscle. Ilizarov (1989a) observed that the muscle fibers became enlarged and noted the formation of myofibrils. Our study showed that the muscle mass was increased. The increase in weight may be due partly to edema, although histological evaluation showed no difference between distracted and non-distracted muscle, and edema would not be likely to contribute in group 3.

Our results show that muscle cell proliferation occurs only during leg lengthening, and confirm that stretch appears to be the major stimulus for longitudinal muscle growth (Ilizarov 1989a). We found no effect on the mitosis rate after 1 week, suggesting that leg lengthening had to be in progress for some period of time or had to reach some extent. The latter is probably the most reasonable interpretation and it corresponds well to signs of myogenesis after a leg lengthening of 10 percent (Paley 1988).

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