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Implantation of autologous cells in minced and devitalized rat skeletal muscles

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Summary

Triceps surae of 3 week-old female Wistar rats were minced and orthotopically autografted. Thirty days later, the regenerates developed a force of 314 ± 58 mN during an isometric maximal tetanus and the maximal area of the muscle tissue was 3.2 ± 0.5 mm^2 (n = 9) in a cross-section. At 60 days, their mean maximal isometric force was increased (1159 ± 367 mN) as also were their maximal muscle cross-section area (6.8 ± 0.7 mm^2; n = 6).

If the minces were devitalized by a freeze and thaw procedure, the regenerates neither contracted under electrical stimulation nor contained any muscle fibre.

If devitalized minces were seeded with myogenic cells isolated from the contralateral triceps surae, orthotopically autografted and allowed to regenerate for 30 days, the regeneration was weak: 46 ± 14 mN of force, 1.2 ± 0.3 mm^2 of muscle area in minces devitalized by cold (n = 7) and 23 ± 8 mN of force, 0.2 ± 0.1 mm^2 of muscle area in minces devitalized by cold supplemented by heat (n = 8).

If viable minces were seeded with myogenic cells, there was no improvement in the extent of regeneration: 275 ± 83 mN of force, 2.0 ± 0.6 mm^2 of muscle area at 30 days (n = 7) and 738 ± 191 mN of force, 5.6 ± 0.9 mm^2 of muscle area at 60 days (n = 5).

Consequently, although it is possible to induce regeneration by grafting myogenic cells into a devitalized mince, this procedure has no effect when applied to a viable mince.

Introduction

If a triceps surae of an adult rat is excised, minced and grafted back into its original site, there occurs a remarkable regeneration (Studitsky, 1964; Carlson, 1968): in a few weeks a regenerated muscle appears, which is vascularized, innervated, contractile and functional, since it is used by the animal during locomotion (Bertrand *et al.*, 1981). However, there is never a complete restoration of the muscle. Even in the best case, the maximal tetanic isometric force remains one third to one quarter of normal. The physiological factors that limit the extent of the regeneration are not well understood (for a recent review, see Plaghki, 1985). However, regeneration is presumably initiated by the satellite cells present in the mince (Snow, 1977; Mayr, 1981) and thus, the initial number of myogenic cells present in the mince might obviously be an important factor.

When all the cells present in a mince are killed by a freeze and thaw procedure, there is no muscle regeneration; if only half of them are killed, regeneration is also halved (Ghins *et al.*, 1984). Therefore, regeneration might well improve if the initial number of myogenic cells is increased in the mince, for example by seeding it with myogenic cells isolated from the contralateral muscle. Such cells survive and are able to form new adult muscle fibres when they are mixed into minces devitalized by freezing and thawing (Ghins *et al.*, 1985), an observation confirmed in the present work. The next step would be to seed a viable mince with myogenic cells.

We know from the literature that satellite cells can differentiate into 'mosaic myotubes' by fusing with autologous myogenic cells *in vitro* (Cossu *et al.*, 1980; Jones, 1982) or *in vivo* (Lipton & Schultz, 1979; Jones, 1979; Watt, 1982; Watt *et al.*, 1982, 1984). Thus, satellite cells present in a mince are presumably able to fuse with autologous myogenic cells isolated from another muscle. We have therefore performed experiments in which viable minces of rat triceps surae are seeded with myogenic cells isolated from the contralateral muscle. Contrary to our expectations, the regeneration is not improved; this observation indicates that the initial number of myogenic cells present in a viable mince is not a factor limiting the extent of muscle regeneration.

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Methods

Surgery

Forty-two female Wistar rats, approximately 3 weeks old, were anaesthetized with 0.2 ml Thalamonal per 100 g body weight, injected subcutaneously. The right triceps surae was excised, minced and orthotopically autografted, as previously described (Carlson, 1968; Ghins *et al.*, 1984). Some of the animals received an additional treatment described below. The regenerates were examined 30 or 60 days after the surgery.

Isolation of cells

The left triceps surae were excised, weighed and finely minced with scissors. The mince was digested for 10 min at 37° C in a gently agitated conical tube (Falcon, 50 ml) containing 3 ml of 0.3% trypsin (pig pancreas, US Biochemical Co.) dissolved in Ham's F12 (Gibco, Europe), and 3 ml of 130 U ml^{-1} collagenase (type Ia, Sigma) dissolved in Ham's F12. The pH of the mixture was adjusted to 7.5. The supernatant was collected and diluted with an equal volume of complete medium (80% Ham's F12; 20% pretested fetal calf serum; Gibco, Europe) in order to inhibit trypsin. The precipitate was further digested twice as described above. The three supernatants were combined and centrifuged (5 min, 800*g*). The cell pellet was suspended in 3 ml of complete medium. Small samples were taken to count the cells in a Neubauer haemocytometer and to initiate *in vitro* cultures. The cells were centrifuged again and the cell pellet was used to seed the mince of the right triceps surae of the same animal (for controls, see below).

Cell cultures

Cells (5000 to 30 000) were plated on collagen coated microwells (diameter 16 mm, Nunclon) or Petri dishes (diameter 50 mm, Nunclon) in complete medium, and cultured at 37° C in a 5% CO_2 humidified incubator (National 3221, Heinicke). The medium was changed every two days, and the cultures were examined daily for at least 10 days.

Treatment of the minces

The animals assigned for 30 days of regeneration were divided into four unequal groups. In the M30 group (M for minced, n = 9), the left triceps surae was excised and discarded; the right triceps surae was minced and orthotopically autografted without any further treatment ('viable' mince). In the MS30 group (M and S for minced and seeded; n = 7), the viable mince of the right triceps surae was seeded with cells isolated from the left triceps surae, and the mixture was grafted into the bed of the right triceps surae. In the MFS30 group (M, F, S for minced, frozen and seeded; n = 7), the mince of the right triceps surae was devitalized by freezing in liquid nitrogen and thawing; then it was seeded with cells and autografted. The MFHS30 group (M, F, H and S for minced, frozen, heated and seeded; n = 8) was similar to the MFS30 group, except that after being thawed, the mince was heated at 65° C for 10 minutes. The animals assigned for 60 days of regeneration were divided into two unequal groups: the M60 group (n = 6) and the MS60 group (n = 5) which were respectively treated as the M30 and MS30 group, except that the duration of regeneration was longer.

Mechanical experiments

The regenerated muscle was exposed, taking care not to damage its blood supply, and its distal tendon was attached to an electro-magnetic ergometer (Maréchal & Plaghki, 1979). It was kept moist and warm by dripping Krebs' solution at 35° C onto it, and stimulated directly by two platinum electrodes. The procedure has been detailed in a previous work (Ghins *et al.*, 1984). After the experiment, the regenerate was dissected out, weighed, fixed in Bouin's solution and stored in formaldehyde 37%.

Histology

Cross-sections 5 μm thick were cut at five levels spaced approximately 4 mm apart, starting 2 mm down from the proximal insertion. They were stained by the Haematoxylin–Eosin–Safran technique. The absolute and relative areas of muscle tissue, fat, fibrous connective tissue and remainder tissue (mostly nerves and blood vessels) were evaluated using a calibrated ocular graticule (E35, Graticules Ltd) mounted in a Leitz ocular (Periplan GF 10 × M) combined with a 20/0.32 objective (Phaco L, Leitz) in a Leitz microscope (Diavert).

Results

Isometric tetanus of regenerating muscle

Typical examples of the maximal isometric tetanus developed by muscles regenerating under various conditions are shown in Fig. 1. The regenerates were tetanically stimulated at different lengths in order to obtain a segment of the force–length relationship. The maximal tetanic isometric force was developed at lengths slightly longer (by approximately 2 to 6%, see Δ 1st, Table 1) than the anatomical reference length L_0, measured with thigh and foot perpendicular to the leg (Bertrand *et al.*, 1981). In most of the cases, the resting force at the optimal length was small, being comprised between 10 and 60 mN. Control experiments have demonstrated that such regenerated muscles can contract repetitively (450 ms tetanus every 4 min) for several hours without showing fatigue. Thus we are fairly confident that the mechanograms shown in Fig. 1 truly reflect the mechanical properties of regenerating muscle. In all cases, we can see that the regenerates develop their force at a normal rate. They maintain the force very well during the isometric plateau and relax rapidly, showing the initial notch typical of the early phase of relaxation. The small modulations visible in some graphs are artefacts caused by some oscillations of the galvanometric device used to record the mechanograms. The only obvious differences between the various graphs are to be found in the magnitude of the force developed by the regenerates.

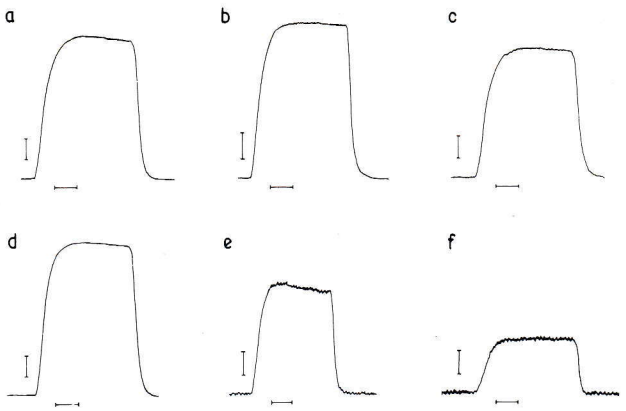

Fig. 1. Maximal isometric tetanus at optimal length (≃ 102% L_o) of perfused regenerating rat triceps surae. Tetanus duration: 450 ms; frequency of stimulation: 100 s^{-1}; mode of stimulation: alternating condenser discharges (time constant 5 ms); temperature: 35° C; horizontal bar: 100 ms. (a) Minced muscle, 30 days after surgery; F_o = 330 mN; resting force: 54 mN; vertical bar: 50 mN. (b) Minced muscle, 60 days after surgery; F_o = 1152 mN; resting force: 51 mN; vertical bar: 200 mN. (c) Minced muscle, seeded with 1.2 million cells, 30 days after surgery; F_o = 178 mN; resting force: not measured; vertical bar: 30 mN. (d) Minced muscle, seeded with 1.3 million cells, 60 days after surgery; F_o: 744 mN; resting force: 30 mN; vertical bar: 100 mN. (e) Minced muscle devitalized by freezing and thawing, and seeded with 1.5 million cells, 30 days after surgery; F_o = 47 mN; resting force: not measured; vertical bar: 10 mN. (f) Minced muscle devitalized by freezing, thawing and heating, and seeded with 1.3 million cells, 30 days after surgery; F_o = 22 mN; resting force: 22 mN; vertical bar: 10 mN.

The maximal tetanic isometric forces (F_0) are reported in Table 1. The regenerates obtained from untreated viable minces (group M30 and M60) develop the largest isometric forces, these being increased approximately fourfold between 30 and 60 days of regeneration. The regenerates obtained from viable minces seeded with approximately 1 to 2 million cells (group MS30 and MS60, Table 1) develop forces that are somewhat *smaller* than those recorded from regenerates of the M30 and M60 groups. However the differences are not statistically significant. It is thus undoubted that, contrary to our expectations, seeding a viable mince with myogenic cells does not improve the extent of regeneration despite the fact that such a seeding procedure induces regeneration in devitalized minces (Ghins *et al.*, 1985; this work, Table 1); however, the developed forces are weak in these groups (MFS30 and MFHS30 groups, Table 1).

The force developed by a muscle depends on its size. As the weight of the regenerating muscle varies according to the treatments (Table 1), we have calculated the corresponding isometric tension as the products of the force and length of a regenerate divided by its weight. The results, P_0 in Table 1, are similar to those observed for the forces.

Table 1 also shows some characteristics of the regenerates and of the cell population used to seed the minces. We isolated approximately 7 to 8 million cells per gram of the donor triceps surae. Nearly all the isolated cells were used to seed a mince (devitalized or not). A sample of the cells was

Table 1. Characteristics of regenerating muscles.

Group	*n*	*Number of isolated cells* (million g^{-1})	*Number of grafted cells* (million)	*Regenerates weight* (mg)	L_o (mm)	Δ 1st (% L_o)	F_o (mN)	P_o (kN m^{-2})
M 30	9	–	0	121 ± 10	20.0 ± 0.4	2.3 ± 0.7	314 ± 58	54 ± 8
M 60	6	–	0	226 ± 21	22.3 ± 0.4	5.3 ± 3.4	1159 ± 367	118 ± 29
MS 30	7	7.1 ± 0.8	1.4 ± 0.2	116 ± 13	21.0 ± 1.4	5.0 ± 2.4	275 ± 83	56 ± 16
MS 60	5	7.2 ± 0.9	2.1 ± 0.2	200 ± 26	21.3 ± 0.9	−0.7 ± 5.5	738 ± 191	80 ± 17
MFS 30	7	7.8 ± 1.8	1.4 ± 0.3	69 ± 4	19.1 ± 0.7	2.6 ± 1.0	46 ± 14	14 ± 5
MFHS 30	8	6.9 ± 1.2	1.3 ± 0.2	59 ± 11	18.4 ± 0.6	1.6 ± 4.1	23 ± 8	7 ± 2

All the data are expressed as the means ± S.E.M.
M 30 and M 60: regenerates obtained from minced triceps surae; 30 and 60 days after surgery.
MS 30 and MS 60: regenerates obtained from minced triceps surae seeded with cells isolated from the contralateral muscle; 30 and 60 days after surgery.
MFS 30: regenerates obtained from minced triceps surae, devitalized by freezing and thawing, and seeded with cells isolated from the contralateral muscle; 30 days after surgery.
MFHS 30: regenerates obtained from minced triceps surae, devitalized by freezing, thawing and heating, and seeded with cells isolated from the contralateral muscle; 30 days after surgery.
L_o: anatomical length of reference (defined in Methods).
Δ 1st: increase (+) or decrease (−) in length required to obtain the maximal isometric force, F_o.
P_o: maximal isometric tension, computed as the product of F_o and corresponding length of the regenerate, divided by its weight.

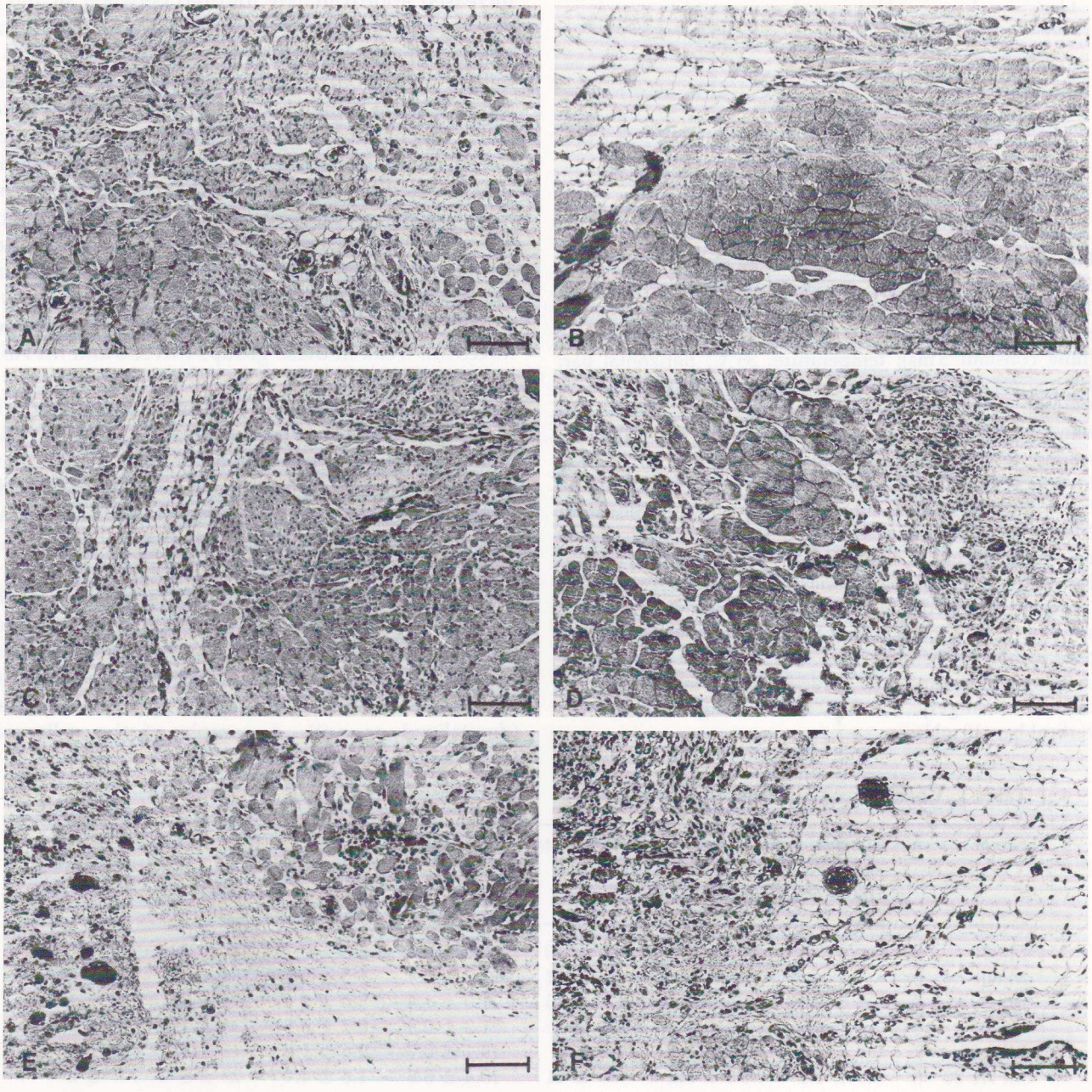

Fig. 2. Cross-sections (6 mm down from the knee), of regenerating muscles at low magnification showing the relative proportions of various regenerated tissues. Horizontal bar: 100 μm; Haematoxylin–Eosin–Safran. (a) Minced muscle; 30 days after surgery; note the presence of numerous muscle fibres showing a number of central nuclei surrounded in some places by dense cellular connective tissue and fat. (b) Minced muscle; 60 days after surgery; muscle fibres are organized in bundles and show few central nuclei; they are very well oriented and there is little nonmuscle tissue. (c) Minced muscle, seeded with cells; 30 days after surgery; the general appearance is similar to that observed in (a) except for a greater proportion of nonmuscle tissue. (d) Minced muscle, seeded with cells; 60 days after surgery; muscle fibres seem less organized than in B and there is more abundant nonmuscle tissue. (e) Minced muscle devitalized by freezing and thawing, and seeded with cells; 30 days after surgery; well and poorly oriented muscle fibres can be seen at upper right, containing some central nuclei; the remaining area is largely occupied by cellular connective tissue and fat. (f) Minced muscle devitalized by freezing, thawing and heating, and seeded with cells; 30 days after surgery; note a large majority of nonmuscle tissue; a few muscle fibres can be distinguished in the lower left corner.

cultured for 10 days. In all these *in vitro* experiments, we observed myotubes on a layer of fibroblasts, with a general appearance similar to that reported in a previous work (Ghins *et al.*, 1985). This demonstrates that myogenic cells were present in all the seeded minces. No attempt was made to evaluate their proportion in the population of isolated cells.

Morphology of regenerating muscle

At the completion of the mechanical experiments, each regenerate was dissected out and processed for classical histology. Sections were made at five levels. Fig. 2 shows typical sections 6 mm down from the knee, a level where muscle fibres are numerous. The general architecture of muscle is well recorded at 60 days (Fig. 2b, d) and more particularly in the M60 group (Fig. 2b). The improvement in organization is evident between 30 and 60 days (compare Fig. 2a and c with Fig. 2b and d). The muscularity of the regenerate is not improved by seeding a viable mince with cells (compare Fig. 2c and d with Fig. 2a and b). Lastly, regenerating muscles obtained from devitalized minces seeded with cells show muscle fibres embedded in large areas of fat or fibrous connective tissue (Fig. 2e, f); muscle fibres are few, particularly in the MFHS30 group (Fig. 2f).

It is remarkable that most of the fibres are transversely sectioned. This fact suggests that nearly all the muscle fibres are well oriented along the axis of the leg. The muscle fibres have normal appearance; only a very few of them are round and eosinophilic, a sign indicating degeneration. The diameter of most muscle fibres at 30 days is approximately 30 µm, but some fibres are as small as 15 µm or as large as 50 µm. At 60 days, the mean diameter is considerably larger and the sizes are less variable than at 30 days. There is no obvious dependence of the diameter of the muscle fibres on the type of treatment. A number of muscle fibres have central nuclei at 30 days. Such immature fibres are occasionally seen at 60 days.

We have done a quantitative analysis of the areas occupied by various tissues in each section. We considered muscle tissue, fat, fibrous connective tissue and the 'other' tissues (mostly nerves and blood vessels). The results are seen in Fig. 3 for each of the six treatments. The area occupied by the fibrous connective tissue (i.e. the distance between filled squares and open triangles in Fig. 3) is approximately 1.5 mm^2 down from the knee until the most distal section. It is reduced to 1 mm^2 in regenerates obtained from seeded devitalized minces, but it is otherwise constant in all other treatments. The area occupied by nerves and blood vessels (ordinates of filled squares in Fig. 3) is approximately equal to 0.5 mm^2; it also remains constant in all situations. Adipose tissue shows a large proximodistally negative gradient which is only

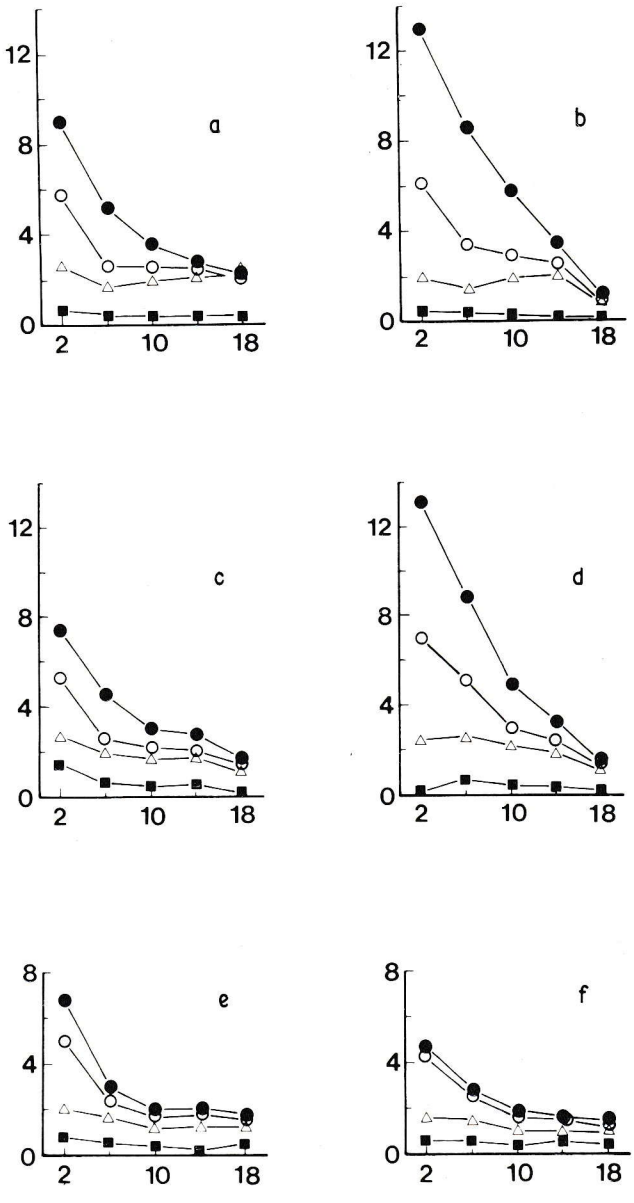

Fig. 3. Tissue composition of regenerating rat triceps surae. Abscissae: distance in mm from the proximal insertion. Ordinates: cross-sectional area in mm^2; ●, total area of the section; the differences between ● and ○ represent muscle fibre areas; between ○ and △, the fat areas; between △ and ■ the fibrous connective tissue areas and the ordinates of ■ represent the areas of other tissues (vessels and nerves). Each point is the mean of n (specified below) experiments. The S.E.M. of the means is not shown, in order not to overload the graph: it comprises between 10 and 25% of the means. (a) Minced muscle; 30 days after surgery (n = 9). (b) Minced muscle; 60 days after surgery (n = 6). (c) Minced muscle, seeded with cells; 30 days after surgery (n = 7). (d) Minced muscle, seeded with cells; 60 days after surgery (n = 5). (e) Minced muscle devitalized by freezing and thawing, and seeded with cells; 30 days after surgery (n = 7). (f) Minced muscle devitalized by freezing, thawing and heating, and seeded with cells; 30 days after surgery (n = 8).

influenced by the duration of the regeneration: in their proximal part, there is approximately twice as much fat (distance between open triangles and open circles) in muscles regenerating for 60 days (Fig. 3b and d) as in those regenerating for 30 days (Fig. 3a, c, e and f). Muscle tissue (distance between open and filled circles) shows the widest variations. First of all, it is distributed along a strongly negative proximodistal gradient. Secondly, the muscle tissue grows between 30 and 60 days (compare Fig. 3a and c with Fig. 3b and d), especially from the proximal insertion of the regenerate up to about 10 mm distally. Thirdly, the muscle tissue is less abundant in the regenerates obtained from devitalized seeded minces (Fig. 3e and f). This is particularly true when the devitalized minces had been further heated before they were autografted (Fig. 3f).

These observations strongly suggest that the regeneration of nonmuscle tissues depends on factors which are independent of those which control the regeneration of muscle tissues.

Relationship between force and muscle tissue

The relationship between the maximal isometric force (F_0, Table 1) and the maximal section area of muscle tissue of the regenerates is shown in Fig. 4. Maximal muscle area was estimated on the basis of the well oriented non-degenerative fibres. It was mostly observed 2 or 6 mm down from the knee. The regression line, computed according to the least rectangles method, has a slope of 208 kN m^{-2}. Another line, passing through the origin and the mean point of all data has a slope of 128 kN m^{-2}. We have drawn a third line, going through the origin with a slope of 315 kN m^{-2}, which is a normal value (Bertrand *et al.*, 1981); it is obvious that such a line is not representative of our data. Thus, the tension developed by the regenerates is obviously less than that measured in untreated muscles. Moreover, the means for each treatment (data not shown) are statistically on the 128 kN m^{-2} line.

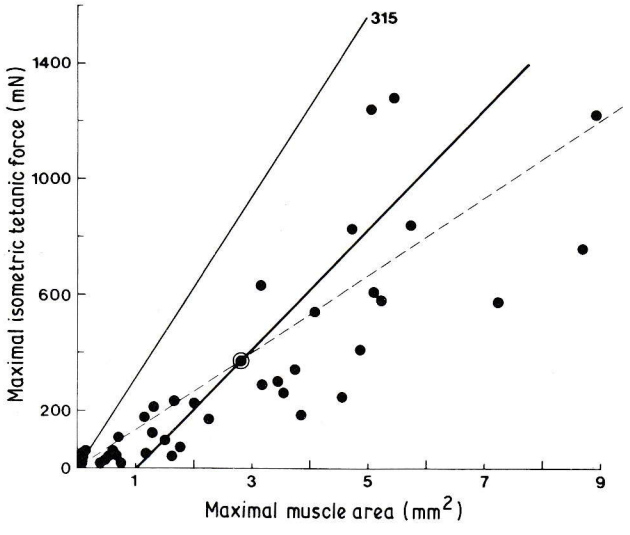

Fig. 4. Relationship between maximal isometric tetanic force and maximal muscle area, only taking into account well oriented and non-degenerated muscle fibres. A regression line, calculated using the least rectangles method, has a slope of 208 kN m^{-2} and starts at x = 1. The point ⊙ represents the arithmetic means of all data: its value is 2.82 mm^2 on the abscissae and 376 mN on the ordinate. The dotted line has a slope of 128 kN m^{-2} and is determined by the origin and the ⊙ point. A third line having a slope of 315 kN m^{-2} (a normal value) is obviously not representative of our data and thus demonstrates that the regenerating muscles develop a maximal tetanic tension weaker than normal. The means of the different treatments (not shown) can be statistically aligned on a straight line similar to the dotted one (slope of 128 kN m^{-2}).

We believe that the weak tension observed for the regenerated fibres is probably explained by a mechanical artefact. In spite of the fact that the regenerate is isometrically tetanized at the optimal length, an internal shortening of the myofibres occurs at the expense of an elongation of the tendons. Since the myofibres are very short and connected to very long tendons, they contract isometrically at a length much shorter than optimal, thereby producing a low tension. In the case of myofibres regenerated in free-grafts, their length is quite normal, and restoration to normal tension is observed after regeneration (Faulkner *et al.*, 1981).

Discussion

Regeneration of striated muscle depends on at least the following factors: presence of myogenic cells, microarchitecture of basal lamina and associated motor end-plates, vascularization, innervation and mechanical stresses (reviews in Carlson & Faulkner, 1983; Plaghki, 1985).

The interpretation of the experiments described in this work requires therefore an evaluation of the relative contributions of these factors. Nevertheless, we believe that our observations can be understood in the framework of a simple hypothesis.

In order to state this hypothesis accurately, we need to recall a few important facts.

Satellite cells located in the endomysial tube inside the basal lamina are activated by lesion of the muscle fibres; they proliferate inside the endomysial tube and fuse to regenerate the damaged muscle fibres (Snow, 1977). They are not able to cross the basal lamina: because of this fact, Bischoff (1975) could observe all the steps of regeneration which occurred inside the endomysial tube of a single fibre in culture. He could also see that the myogenic cells spilled into the surrounding medium if the tube was broken. In this case, the regeneration appeared to be impaired.

When muscle fibres are selectively killed by a myotoxin, such as a local anaesthetic (Bupivacaine) or

a snake toxin (taipoxin), the architecture of basal lamina and endomysial tube is preserved, as well as the vascularization, whilst the re-establishment of a functional innervation is extremely rapid (Harris & Maltin, 1982). Since satellite cells are resistant to the myotoxic drugs, they proliferate and rapidly restore a muscle which appears morphologically normal (Hall-Craggs, 1980; Maltin *et al.*, 1983).

Finally, myoblasts which are injected under the muscular aponeurosis may wander for long distances in the extracellular spaces, outside the endomysial tube; eventually, a proportion of them cross the basal lamina and fuse within a myofibre (Lipton & Schultz, 1979).

These experiments strongly suggest the hypothesis that muscle regeneration depends on an interaction between myogenic cells and basal lamina, an hypothesis previously proposed in cases of regeneration following local injuries (Vracko & Benditt, 1972). If the myogenic cells remain in the endomysial tube, the regeneration may be fast and complete (depending then on the other factors cited above). If the myogenic cells are located in spaces outside the endomysial tubes, they may cross the basal lamina of neighbouring muscle fibres or collide with other wandering myoblasts. However the chances are high that the wandering myoblasts are lost and do not contribute to the muscle regeneration.

The interpretation of our experiments is clear in the light of these ideas.

In minced muscle, the longitudinal continuity of the basal lamina is destroyed, but there remain large portions of endomysial tubes and basal laminae even though the myofibres degenerate and are phagocytosed; the myoblasts proliferate and fuse inside the old basal laminae. During the early phase of the regeneration, many myoblasts may escape through the cut ends of the endomysial tubes and thus become inefficient for muscle regeneration. Another factor, specific to the minced muscle model, is that most of the old endomysial tubes are not oriented properly in the field of mechanical forces. Their orientation is corrected quite early in the regeneration, within three or four weeks after surgery (Carlson, 1972; this work, Fig. 2). It is not known whether the endomysial tubes rotate to align properly in the mechanical field, or whether they are destroyed and rebuilt in the new direction. These factors seem powerful enough to account for the weak regeneration observed in minced triceps surae (20 to 30% of normal).

When minced triceps surae is seeded with the cells isolated from the contralateral muscle, the initial number of myogenic cells present in the mince should be doubled, and, yet, the regeneration is not improved (Table 1). This can be explained if, when added externally to the endomysial tubes, the myogenic cells are unable to contribute noticeably to the regeneration which occurs within these tubes.

When minced muscle is devitalized by a freeze and thaw procedure, there is no muscle regeneration (although there is regeneration of nonmuscle tissue, Ghins *et al.*, 1984). The reason is that the procedure has killed all the myogenic cells which were initially present in the mince. When the initial number of myogenic cells is restored by seeding the devitalized mince with the cells isolated from the contralateral muscle, there occurs a muscle regeneration, but it is very weak (4% of normal). Apparently, the myogenic power of the cells is much impaired if they are located in the extracellular spaces, outside the endomysial tubes.

When a minced muscle is divided into two equal portions, one of which is devitalized by a freeze and thaw procedure, the mixture of the two portions contains a number of myogenic cells which is approximately half that contained in an entire viable mince. In this case, the regeneration is approximately half that observed in the case of an entire viable mince (Ghins *et al.*, 1984). Thus, the regeneration is noticeably stronger than in the case of a devitalized mince seeded with a full complement of myogenic cells. The reason for the improvement of regeneration might be that the myogenic cells are still encased in the remains of the old basal laminae and are thus able to express their full myogenic power.

We are aware that there are alternative hypotheses to the one discussed above.

For instance, we suppose that we are able to isolate all the myogenic cells in good condition. It may be that most of these have lost their myogenic power, or some property needed to interact with other cells or basal lamina. The remaining cells which are competent for myogenesis, would therefore be few in number. There would be no need to hypothesize some differences between myoblasts according to their situation vis à vis the basal lamina in order to explain the little success of seeded cells in inducing regeneration. We believe that in the present state of knowledge, it is impossible to answer this objection.

Regeneration could also be limited by the proliferating power of the vascular tissue, since it is known the capillary:muscle fibre ratio is depressed in transplanted muscle (White *et al.*, 1981). This could explain why regeneration of minced muscle is not improved by increasing its content of myogenic cells, but we do not see how this hypothesis could account for the very weak regeneration of devitalized mince seeded with a normal complement of myogenic cells.

Reinnervation is also a factor of importance in the regeneration of muscle (Carlson, 1973; Faulkner *et al.*, 1981). Many experiments have shown that the integrity of the basal laminae, especially at the motor end-plates, is essential for a successful innervation

(Marshall *et al.*, 1977; Carlson & Faulkner, 1983; Ecob, 1984). Bader (1980) has reported that free grafts without motor end-plates regenerate very poorly. The neural hypothesis might explain the outcome of our experiments, the more so in that several aspects of it are variations of the basal lamina hypothesis discussed above. However, it is not satisfactory for the following reasons. The early phase of regeneration, up to the stage of maturing myotubes, occurs in complete independence of innervation. This is true for the free grafts without end-plates of Bader (1980). It has also been clearly demonstrated in the regeneration of sliced muscle grafts (Carlson & Gutmann, 1976): their regeneration (in the rat) is identical in the denervated or innervated leg, up to 30 days after surgery; at 60 days the regeneration has much improved in the innervated leg, but is strongly depressed in the denervated leg. (Note that the durations of regeneration correspond with those used in the present work.) If a proper innervation was the limiting factor in our experiments, we should have observed (1) that minces seeded with myogenic cells regenerate much better than ordinary minces up to 30 days after surgery, but lose their advantage at 60 days after surgery, and (2) that devitalized minces seeded with cells regenerate as well as ordinary minces.

Concerning the mechanical stress, we have no information to evaluate its possible effect on cells present in devitalized seeded minces and in viable minces.

In conclusion, our experiments support the hypothesis that the myogenic power of myoblasts is severely impaired if the cells are located outside the basal lamina.

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