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The origin of muscle stem cells in rat triceps surae regenerating after mincing

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Summary

Rat triceps surae was minced and orthotopically autografted. Twitch time to peak, maxima tetanic tension, lactate dehydrogenase activity and total creatine concentration were measured in muscles regenerating for 30, 60 and 90 days.

If the minces were frozen and thawed before grafting, muscle regeneration was suppressed. If they were further heated before grafting, muscle regeneration was also suppressed. If one half of the mince was either frozen and thawed or frozen, thawed and heated, and then recombined with the remaining half, muscle regeneration was delayed. However, at 90 days, 'intensive properties' (twitch time to peak, maximum tetanic tension, total creatine concentration and lactate dehydrogenase activity) of regenerates obtained from such partially treated minces were similar to those of regenerates obtained from untreated minces although their 'extensive properties' (weight and maximal tetanic force) were approximately halved.

The extent of regeneration depends on the mass of untreated mince autografted and thus, presumably, on the number of viable muscle stem cells initially present in the mince.

Introduction

Mammalian skeletal striated muscles are endowed with a powerful capacity to regenerate, as demonstrated by Carlson in 1968. In his experiments, rat triceps surae muscles were excised, minced and grafted back in their original site. Necrotic muscle fibres are phagocitized by macrophages brought into the grafts with invading capillaries within a few days after surgery. At this time, mononuclear cells appear, proliferate and fuse into myotubes. A new muscle matures (Carlson, 1973), is reinnervated (Bennet *et al.*, 1974) and its physiological characteristics slowly improve with time (Bertrand *et al.*, 1981).

The origin of the myogenic cells is still controversial. Most authors are of the opinion that these cells come from activated satellite cells that have survived the mincing procedure (Mayr, 1981), although some role of myonuclei or mesenchymal cells cannot

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be excluded (Aloisi & Mussini, 1981). Myogenic cells could also be brought into the mince by the blood circulation, or invade it by creeping from neighbouring muscles (Sloper & Pegrum, 1967; Partridge & Sloper, 1977; Grounds *et al.*, 1980; Sloper & Partridge, 1980; Allbrook, 1981; Jockusch *et al.*, 1983). The extent of this colonization appears to be fairly variable, depending on the experimental conditions. In at least one case, its occurrence could be excluded (Cosmos *et al.*, 1979). There are also some indications that the contribution of colonizing cells is negligible in the rat minced muscle model (Carlson, 1972), but we know of no systematic work on this problem.

We report in this paper that regeneration of minced muscle is completely inhibited if all the cells of the mince are killed by a freeze and thaw procedure. Heat inactivation of enzymes released by thawed cells does not restore regeneration. If one half of the cells is submitted to these procedures, regeneration occurs but is depressed. However, all properties are not depressed to the same extent. The properties that depend on the mass of the regenerate ('extensive properties'), such as weight or maximal tetanic force, are much more depressed than the properties that are independent of the mass of the regenerate ('intensive properties'), such as twitch time to peak, maximum tetanic tension, concentration of substrates and enzyme activities. These experiments indicate that the overwhelming majority of muscle stem cells originates from the wounded muscle, at least in minced rat triceps surae.

Methods

Mincing procedure

The experiments were performed on 66 female Wistar rats of an outbred local stock obtained from the Katholieke Universiteit te Leuven. All animals were one month old at the time of surgery. The mincing procedure was similar to that described by Carlson in 1968. Anaesthesia was induced by 0.2 ml s.c./100 g body weight of Thalamonal (Janssens Pharmaceutica). The right leg was shaved and cleaned with ethyl alcohol. The whole surgery was performed under a stereoscopic zoom microscope (SMZ, 2 type 3 Nikon) equipped with a remote-control device. A long latero-external incision was made, first through the skin, then through the biceps femoris. The triceps surae was dissected out, taking care to preserve sural and common peroneal nerves and sural artery and vein. The tibial nerve, the middle and lateral inferior genicular arteries, together with the internal and external sural arteries going to the triceps surae were dissected from the hilum well into the muscle, as far as possible. The muscle was brought into a sterile Petri dish and cleaned of its tendons. The soleus was discarded. The remainder of the triceps surae was finely cut with scissors ('minced') and submitted to one of the treatments described below. The mince was grafted back into the leg without any particular disposition. Biceps femoris and skin were separately sutured (Ethicon 6/0).

The rats were kept in individual cages in an air-conditioned room lit with cycling artificial lights.

Treatments of the minces

The rats were arranged in five unequal groups. In group M (minced), the triceps surae was minced and grafted as described above. The mincing procedure destroys muscle fibres but spares the small mononucleated cells present in the muscle. In group MF (minced and frozen), the mince was frozen in liquid nitrogen and thawed before being grafted. Such a treatment kills all the cells by rupturing their membranes. In group MFH (minced, frozen and heated), the mince was frozen in

liquid nitrogen, thawed and heated for 10 min at 65° C; then it was grafted into the animals. The heating treatment presumably abolishes some toxic environment created by the freeze and thaw procedure (see Discussion). In group MF/M, half of the mince received treatment MF; then it was thoroughly mixed with the remaining half (treated as M) and grafted. In group MFH/M, half of the mince received treatment MFH and was mixed with the remaining half (treated as M) before being grafted. The grafts were left to regenerate for 30, 60 and 90 days. Two animals died a few weeks after surgery for unknown reasons and eight were discarded at the time of mechanical experiments owing to technical difficulties encountered with the ergometric equipment.

Mechanical experiments

The animal was anaesthetized with s.c. Thalamonal. The regenerate was dissected, beginning from its distal end upwards towards its proximal end. Great care was taken to preserve the blood supply going proximally into the regenerate. The distal end was sutured to a light silver chain, then cut and attached to an ergometer (Maréchal & Plaghki, 1979). The regenerate was maintained by continuously dripping Krebs solution (34° C; pH 7.4) onto it. Two fine platinum electrodes (diameter 0.2 mm) were laid on the belly of the regenerate. The reference length l_0 was measured with thigh and foot perpendicular to the leg (Bertrand *et al.*, 1981). Twitch time to peak was measured at optimal length after a series of a few twitches. The regenerate was tetanized with slightly supramaximal condenser discharges (5 ms time constant; 100 s^{-1}) for 450 ms. A portion of the force–length relation was obtained in order to evaluate the maximum isometric tetanic force. The corresponding maximum tension was computed as the product of maximum force, length and water density divided by the weight of the regenerate: it was expressed in units of force per area, kN m^{-2}. Intervals between the tetanic contractions lasted 4.5 min in order to minimize fatigue.

Chemical analyses

At the end of the mechanical experiment, the regenerate was dissected out, weighed, frozen in liquid nitrogen and kept at −90° C until use. The regenerate was extracted on ice in about 100 vol. 80 mM KCl, 0.1 mM dithiothreitol, 1 mM TES buffer (pH 7.0). Total creatine (sum of creatine and phosphocreatine) was measured by the α-naphtol diacetyl procedure (Ennor & Stocken, 1948), together with the lactate dehydrogenase activity (Bergmeyer & Bernt, 1970) and proteins, separated in soluble and insoluble fractions (Lowry *et al.*, 1951). No chemical analyses were performed on the regenerates of the MFH group.

Clinical observation

At the end of the regeneration period, the progress of regeneration was assessed by a clinical examination (except for group MFH), using criteria described in Bertrand *et al.* (1981).

Results

Mechanical experiments

One of the most appropriate means of assessing levels of functional return in regenerating muscle is the maximum force developed during a fused tetanic contraction at optimal length. All the regenerates from standard minces (group M) contract noticeably (Table 1). Their force increases as regeneration proceeds, doubling for every period of 30 days after surgery. At 90 days after surgery, the maximum isometric force equals 1110 ± 98 mN. It is interesting to compare this regenerated force with that of intact muscle. We have not measured the isometric maximum force in the controlateral

Table 1. Mechanical characteristics of regenerates.

Group	n	*Duration of regeneration* (days)	*Weight of regenerates* (mg)	*Maximum isometric force* (mN)	*Maximum isometric tension* (kN m^{-2})	*Twitch time to peak* (ms)
M	6	30	120 ± 12	257 ± 45	49 ± 12	40 ± 1
	4	60	139 ± 3	677 ± 108	106 ± 14	30 ± 2
	5	90	221 ± 8	1110 ± 97	125 ± 14	24 ± 3
MF	4	30	31 ± 19	0	0	—
	4	60	45 ± 17	0	0	—
	4	90	57 ± 4	0	0	—
MFH	6	30	51 ± 3	0	0	—
MF/M	4	30	117 ± 11	76 ± 11	16 ± 3	44 ± 3
	3	60	109 ± 9	181 ± 85	42 ± 7	26 ± 2
	3	90	182 ± 22	572 ± 128	79 ± 26	28 ± 2
MFH/M	4	30	110 ± 8	196 ± 56	38 ± 10	45 ± 4
	6	60	128 ± 12	383 ± 63	63 ± 8	34 ± 1
	3	90	162 ± 16	723 ± 138	115 ± 24	27 ± 1

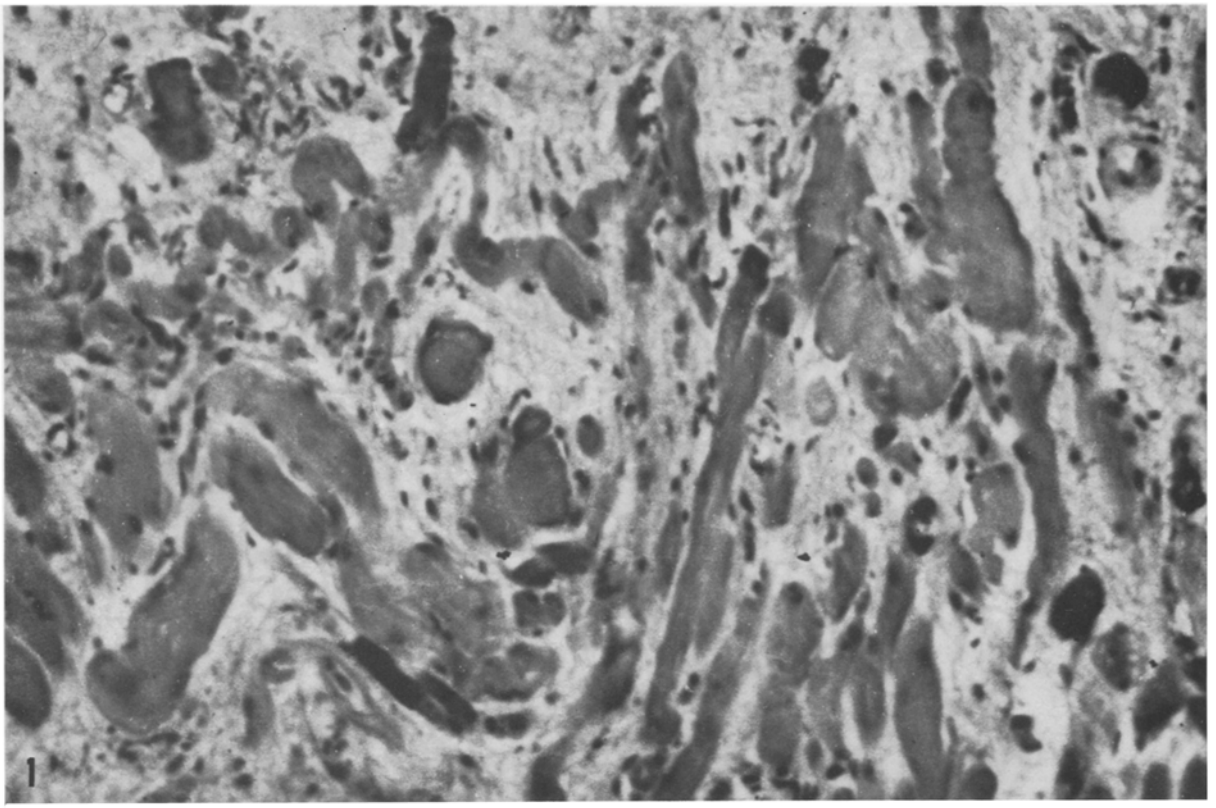
All data are expressed as the mean ±S.E.M. The maximum isometric forces are expressed in millinewtons (mN) and the maximum isometric tensions in kilonewtons per square metre (kN m^{-2}).

muscle, but we have measured the force of intact triceps surae in a few animals of similar size: we have observed a maximum isometric force approximately ten times larger than that of 90 days regenerates. The force deficit observed in regenerates is explained by two factors. Firstly their weight is smaller by two-thirds than that of normal muscle, and secondly their tension (force per unit cross-section) is also smaller by two-thirds. Twitch time to peak decreases as regeneration proceeds (Table 1); as this parameter is a rough evaluation of the velocity of contraction, this result indicates that ageing regenerate becomes faster. All these results are in good agreement with earlier reports (Carlson & Gutman, 1972; Plaghki *et al.*, 1980a; Bertrand *et al.*, 1981).

If muscle mince is devitalized by cold (group MF) or by heat combined with cold (group MFH), the regenerate is small in weight and never contracts under electrical

Fig. 1. Cross-section through a 30 day contractile regenerate (group M). Numerous muscle fibres of various diameter, embedded in a dense cellular connective tissue. The section shows a region where the fibre architecture is disorganized. Elsewhere most fibres are axially oriented. A few fibres have central nuclei. 5 μm. Masson's trichrome stain. × 320.

Fig. 2. Cross-section through a 60 day noncontractile regenerate (group MF). Numerous nerves, vessels, fat and connective tissue are seen but there are no muscle cells. 5 μm. Masson's trichrome stain. × 490.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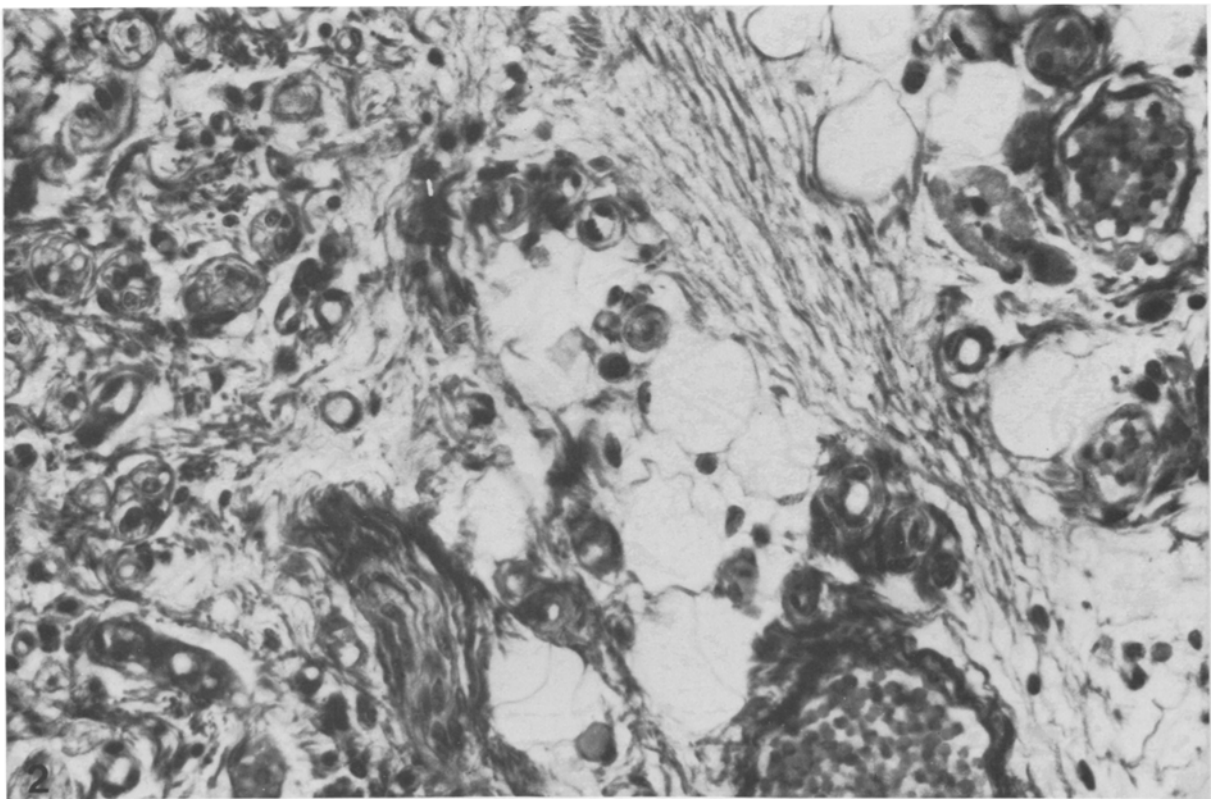
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stimulation. The limit of force detection (1 mN) is 1/10 000 of the maximum tetanic force of intact muscle, or 1/1000 of the maximum tetanic force of the strongest regenerate. A few histological studies confirmed the total absence of muscle fibres in cross-sections of noncontractile regenerates, although young muscle fibres were numerous in contractile regenerates (Figs. 1 and 2).

Graft of normal mince combined with frozen (group MF/M) or frozen and heated (group MFH/M) mince regenerates muscle with intermediate functional values for maximum tetanic force and weight ('extensive properties'), whilst maximum tetanic tension and twitch time to peak ('intensive properties') are less or not modified (cf. Table 1).

Biochemical measurements

Direct measurement of contractile proteins would offer the best biochemical mean for assessing the extent of muscle regeneration. A very rough estimate is obtained from the measurements of protein insoluble in buffered 80 mM KCl (fractions NS, Table 2). This fraction is obviously much lower in regenerate from devitalized mince (group MF). Soluble protein (fraction S), which includes mainly protein dissolved in cytoplasm, is somewhat higher in group MF and MF/M. We have not performed more accurate measurements of contractile proteins, since they are quite difficult to do quantitatively. We have tried to estimate the density of functional muscle tissue in regenerate from

Table 2. Biochemical characteristics of regenerates.

Group	n	*Duration of regeneration* (days)	*S protein* (mg g^{-1})	*NS protein* (mg g^{-1})	*Total creatine* (μmol g^{-1})	*LDH* (IU g^{-1})
M	6	30	44 ± 1	25 ± 2	5.8 ± 0.7	85 ± 10
	4	60	43 ± 1	34 ± 8	7.6 ± 0.9	152 ± 25
	5	90	45 ± 1	35 ± 3	9.9 ± 0.5	194 ± 7
MF	4	30	40 ± 24	8 ± 6	0.7 ± 0.4	20 ± 12
	4	60	53 ± 19	6 ± 2	1.2 ± 0.4	23 ± 11
	4	90	60 ± 8	13 ± 4	1.2 ± 0.4	14 ± 6
MF/M	4	30	56 ± 2	24 ± 2	4.2 ± 0.8	69 ± 13
	3	60	56 ± 2	22 ± 6	4.6 ± 1.3	110 ± 31
	3	90	63 ± 4	30 ± 7	6.9 ± 1.1	166 ± 19
MFH/M	4	30	39 ± 2	20 ± 2	4.2 ± 0.4	54 ± 5
	6	60	47 ± 3	13 ± 2	3.9 ± 0.2	76 ± 7
	3	90	49 ± 4	20 ± 6	7.5 ± 1.5	145 ± 28

All data are expressed as the mean ± S.E.M. per gram of fresh tissue. S protein, fraction of proteins soluble in 80 mM KCl; NS protein, fraction of proteins insoluble in 80 mM KCl; LDH, lactate dehydrogenase activity. Total creatine, sum of creatine and phosphocreatine.

measurements of two parameters that are strongly correlated to it: total creatine (sum of creatine and phosphorylcreatine) concentration, and lactate dehydrogenase (LDH) activity. Total creatine is proportional to the maximum energy store available for alactacid anaerobic contractions. It is also proportional to muscle tension (Wilkie, 1968) and to actin concentration (Plaghki *et al.*, 1980a). Regenerate from untreated mince has a sizeable concentration of total creatine, which increases with the duration of regeneration. Its highest value (9.9 μmol g^{-1}), observed 90 days after surgery, is equal to approximately a third of that typical for intact muscle (33 μmol g^{-1}, Plaghki *et al.*, 1980b). The deficit in total creatine concentration is thus in good agreement with that of maximum tetanic tension, which is also depressed to a third of the normal value.

Regenerate from devitalized mince (group MF) has low but measurable total creatine and regenerate from mixture of devitalized and viable mince has intermediate total creatine content. These results are in good agreement with tension, and suggest a close correlation between the two parameters. Regression analysis of total creatine on maximum tetanic tension was computed on data combined of all groups. It shows that maximum tetanic tension increases by 13 kN m^{-2} for every increase of 1 μmol g^{-1} of total creatine ($r = 0.77$; $n = 38$; $P < 0.001$). This relation can be used to predict by extrapolation the maximum tetanic tension of intact muscle, knowing its total creatine concentration (33 μmol g^{-1}). It turns out to be equal to 420 kN m^{-2}, a result in excess of the observed value (315 kN m^{-2}). It suggests that the yield of tension per total creatine is somewhat better in regenerating muscle than in normal intact muscle. The regression line intercepts the total creatine axis for a total creatine concentration of 1.2 μmol g^{-1}. This is expected since regenerate from devitalized mince has a small concentration of total creatine, but does not contract. This creatine is probably located in nonmuscle cells.

Lactate dehydrogenase is very active in muscle fibres, especially of type 2. Although not specific for muscle fibres, since it is present in many cells, it still is a fairly good indicator for muscle. After surgery regenerate from viable mince (group M) has LDH activity increasing with a time course very similar to that of total creatine and maximum tension. Its activity 90 days after surgery (194 IU g^{-1}) is equal to one-third of that observed in intact muscle (600 IU g^{-1}), in agreement with earlier observations (Plaghki *et al.*, 1980b). Regenerate from devitalized mince (group MF) has low lactate dehydrogenase activity, probably localized in the mononuclear cells of various kinds present in it. Regenerates from mixture of devitalized and viable mince have intermediate lactate dehydrogenase activity.

These observations suggest a correlation between lactate dehydrogenase activity and maximum tension, a correlation to be expected from the nature of these parameters. Regression of lactate dehydrogenase activity on maximum tension using data of all groups indicates an increase of 0.6 kN m^{-2} for every increase of 1 IU g^{-1} of LDH activity ($r = 0.76$; $n = 38$; $P < 0.001$).

Clinical observations

A third way to evaluate regeneration might be based on clinical observations. This

would be valuable in that observations of such type might indicate the functional value of regenerate for the animal. We have used a method reported by Bertrand *et al.* (1981) that consists of two types of evaluation. In the first type one observes the shape of the foot and its position relative to the leg. Four stages are defined. Stage 1 is the normal one. Stage 4 is the worst one, observed right after surgery. During regeneration, animal changes from stage 4 back to stage 1, going through stages 3 and 2. Complete recovery to stage 1 occurs only in a few animals. In the second type of measurements one measures the extension deficit defined as the angle between the normal fully extended position of the foot and the actual position attained in the operated limb by a forced extension. Further details may be found in the report of Bertrand *et al.* (1981). Our results for standard mince (group M, Table 3) are quite similar to those of Bertrand *et al.* (1981). However, we observed noticeable clinical recovery (back to stage 2, cf. Table 3) in animals grafted with devitalized mince (group MF) that neither contracts (Table 1) nor has a sizeable total creatine content or lactate dehydrogenase activity (Table 2), two parameters that are high in normal muscle.

Rats grafted with mixed mince (groups MF/M and MFH/M) clinically recover much like those grafted with devitalized mince (Table 3). Our experiments indicate that much of the clinical recovery described by Bertrand *et al.* (1981) is not dependent on muscle regeneration.

Table 3. Clinical parameters of regeneration.

Group	n	*Duration of regeneration* (days)	*Body weight* (g)	*Clinical stage*	*Angular deficit* (degree)
M	6	30	154 ± 5	2.8 ± 0.1	22 ± 1
	4	60	181 ± 3	1.6 ± 0.4	22 ± 3
	5	90	214 ± 6	1.4 ± 0.2	14 ± 2
MF	4	30	153 ± 11	2.5 ± 0.1	23 ± 3
	4	60	195 ± 11	2.0 ± 0.2	30 ± 3
	4	90	221 ± 8	2.0 ± 0.4	18 ± 3
MF/M	4	30	148 ± 3	2.9 ± 0.1	23 ± 2
	3	60	195 ± 11	1.7 ± 0.3	24 ± 6
	3	90	215 ± 11	2.0 ± 0.1	19 ± 5
MFH/M	4	30	153 ± 5	2.4 ± 0.2	26 ± 2
	6	60	193 ± 4	1.8 ± 0.1	27 ± 2
	3	90	209 ± 4	2.0 ± 0.1	31 ± 2

All the data are expressed as the mean ± S.E.M. Body weight refers to the weight of the animals at the end of the regeneration period. The angular deficit is expressed as a lack of extension of the foot on the leg and its value is zero in unoperated animals. Clinical stages are described in text and further in Bertrand *et al.* (1981).

The possibility that treatments did not depress the growth rate of rats was checked. Body weight is a useful parameter for that purpose. It increases similarly in all groups after surgery (data not shown) and equals that predicted by growth charts at the end of the regeneration period (Table 3).

Discussion

The mincing procedure destroys the muscle fibres and disrupts the connective organization of vascular and nervous tissues. It does not destroy the smaller mononuclear cells (fibrocytes, endothelial cells, satellite cells). Survival of such cells is demonstrated by the success of culture of cells obtained from minces of adult muscles (Bischoff, 1974). However it is also conceivable that similar cells could be brought into regenerates from blood or surrounding muscles, i.e. have an exogenous origin (Grounds *et al.*, 1980). Our experiments were designed to evaluate the respective role of exogenous cells with respect to local or endogenous cells in the regeneration of muscle.

A first group of experiments is used as a control (group M). In these experiments, as the regeneration proceeds, we observe twitch time to peak, maximum tetanic tension, total creatine and lactate dehydrogenase activity changes that are similar to those reported in earlier works (Bertrand *et al.*, 1981; Carlson & Gutmann, 1972; Plaghki *et al.*, 1980a, b).

If the mince is frozen and thawed (group MF) before being grafted, regenerates do not contract and have low levels of insoluble proteins (mostly contractile proteins), lactate dehydrogenase activity and total creatine. The freeze and thaw procedure destroys all the small mononuclear cells that have survived the mincing procedure. This statement is supported by the complete absence of cells in cultures started from such treated minces (unpublished experiments). The vascular invasion thus brings into the regenerates not only capillaries but fibroblasts and adipoblasts as well. It does not however bring any stem cells able to regenerate muscle tissue. Thus stem cells responsible for muscular regeneration in control minces must be present in the mince itself (endogenous origin). They are presumably satellite cells (Mayr, 1981).

The following objection might be raised to this interpretation. It could be that muscle stem cells are effectively of exogenous origin, and thus are able to seed minces and regenerate muscle tissue in the original model of Carlson, but they would not survive in frozen and thawed minces owing to some toxic environment created by the cellular debris. To be consistent with this hypothesis, one should accept that endothelial, fibroblastic and even nervous cells are more resistant to the supposed toxic environment; this is not impossible, although rather unlikely. We performed the following further experiments to test the validity of this objection.

In a first test of this objection, we tried to suppress the postulated toxic environment. It seems reasonable to hypothesize that this toxic environment is due to large molecules, presumably proteolytic enzymes, rather than to small molecules, since we know that such molecules as ATP and creatine are rapidly washed away by diffusion from the

regenerates (Plaghki & Maŕechal, 1976). As many proteins and enzymes are heat labile, we submitted frozen and thawed minces to heat treatments (group MFH); this did not restore muscle regeneration. However this experiment is not quite conclusive, since it can be argued that the hypothetic toxic environment is resistant to heat.

In a second test of the objection, we submitted two halves of the muscle to different treatments: one was minced in the usual way, the other was minced, frozen and thawed. Then we mixed the two halves thoroughly (group MF/M). If the frozen and thawed half was toxic for muscle stem cells, it was expected that it should inhibit regeneration when mixed with an untreated half. It was not the case; regeneration was indeed depressed but not suppressed, indicating that local muscle stem cells survived. Moreover, at a time when the 'extensive properties' (weight and maximum tetanic force) were markedly depressed, the 'intensive properties' (maximum tetanic tension, twitch time to peak, lactate dehydrogenase activity, total creatine) were much less so, especially when the regenerates were at their maximum level of regeneration (90 days). These results suggest that the quantity of regenerated muscle is dependent on the initial number of muscle stem cells, but that its quality is not.

If the frozen and thawed half is further submitted to a heat treatment (group MFH/M), 'extensive properties' are slightly improved at each time of regeneration, indicating that heat treatments inhibit somewhat the depressive effect of the freeze and thaw procedure. The general evolution of the three groups of contractile regenerates is that MFH/M and particularly MF/M groups are delayed in comparison with the M group and that this slowness is less and less marked as regeneration proceeds (at least for the 'intensive properties'). Perhaps it reflects the great difficulty that cells encounter for realizing connections, proliferating and fusing into myotubes at the beginning of the regenerating processes. With respect to 'extensive properties' their magnitude is a function of the mass of untreated mince autografted and thus presumably of the number of muscle stem cells initially present in the grafts.

This assertion is at variance with that of earlier reports (Sloper & Pegrum, 1967; Partridge & Sloper, 1977; Grounds *et al.*, 1980; Sloper & Partridge, 1980; Allbrook 1981; Jockusch *et al.*, 1983). An explanation for this discrepancy is probably to be found in the different species used and the smaller size of the regenerating tissues, since all the experiments of these authors were made on very small muscles of mice. As we know that satellite cells are able to migrate over short distances (Lipton & Schultz, 1979), it is conceivable that their contribution from neighbouring tissues might be appreciable in mouse regeneration model. Indeed, as it was previously reported (Grounds *et al.*, 1980), neighbouring muscles were at times injured during surgery. It is thus possible that the resulting activation of satellite cells in these muscles might support some exogenous colonization which would appear noticeable in mouse muscle, owing to its thinness, but negligible in rat muscle, since this is so much larger. We conclude from our experiments that regeneration of muscle fibres, in the minced rat muscle model, requires muscle stem cells present in the muscle, very few of them, if any, being brought into the mince from outside.

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