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Studies on muscle fibre splitting in skeletal muscle

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Summary. Autoradiographic, stereological and histological studies have been carried out to determine the origin of muscle fibre splitting which supposedly occurs during muscle hypertrophy. The results obtained clearly indicate that the supposedly 'split fibres' are a transient response probably derived from satellite cells and are not derived from pre-existing fibres by true splitting. Similarly, increases in muscle fibre size are not achieved by recruitment of satellite structures as indicated by lack of myonuclear recruitment.

Muscle fibre splitting has traditionally been regarded as a frequent non-specific feature of many chronic muscle diseases, notably in the muscular dystrophies. Its implications have attracted little attention though its biological importance has recently been emphasized[2,3]. Numerous reports have also indicated that severe exercise or muscular hypertrophy is associated with the development of similar but transient histological changes that are usually interpreted, though without evidence, as fibre splitting[4–12]. The present electron microscopical, stereological and autoradiographic study was undertaken to establish whether such a view is tenable.

Adult male mice weighing 20–25 g were randomly selected and subjected under sterile conditions and ether anaesthesia to a unilateral myectomy of the left tibialis anterior muscle of the hindlimb. It is known that removal of this muscle induces hypertrophy and fibre splitting in the ipsilateral extensor digitorum muscle[10,13,14]. During the operation great care was taken not to disturb any of the EDL muscles or their neurovascular supply. A sham operation, in which the tibialis anterior muscle was undisturbed, was carried out on the contralateral limbs of approximately one half of each experimental group of animals. The mice were allowed to recover for varying periods post-operatively prior to examination of their EDL muscles. It is well established that, even where deliberate trauma has been inflicted on rodent muscle sufficient to induce necrosis of a substantial proportion of fibres, satellite cell repair activities cease within 9 days[15].

At 7, 18, 35, 68, 98 and 128 days post-operatively specimens of 36 ipsilateral EDL muscles were obtained for electron microscopy[16,17] and enzyme histochemistry using routine procedures previously described[11,14]. Thick fresh frozen cryostat sections were also obtained which were stained to demonstrate succinate dehydrogenase activity (E.C.1.3.99.1). Thin sections of normal and hypertrophic EDL muscles were examined with a Philips 200 electron microscope during the early phases when the first occurrence of fibre splitting was expected to be most readily visible. The satellite cells clearly became most active at 7 and also at 18 days post-operatively as indicated by their increased electron lucency, by the formation of a distinct Golgi apparatus, by the occurrence of more numerous mitochondria and a marked reduction in the amount of peripheral nuclear heterochromatin (figure 1). The formation of satellite structures which were defined as those larger structures possessing clearly visible myofilaments and which lay in the same topographical relationship to

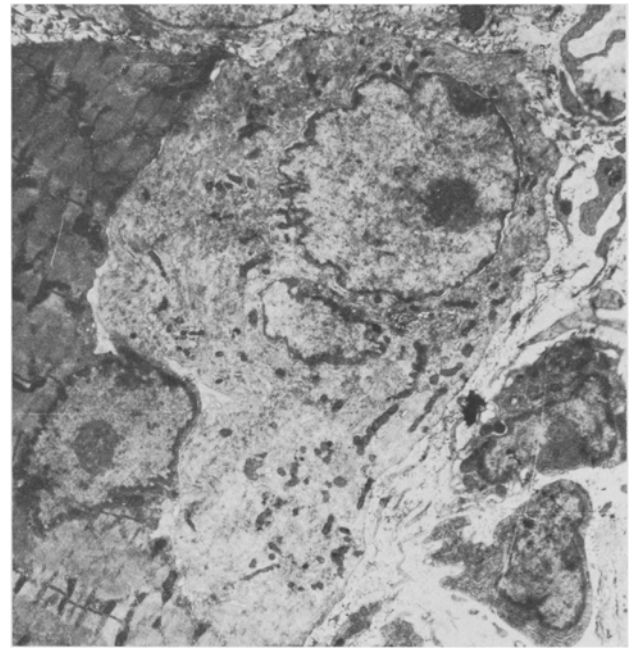

Fig. 1. Electron micrograph of satellite cell in 18-day hypertrophic EDL muscle. The cytoplasm is markedly electron lucent and is clearly increased in amount. The nucleus possesses a prominent nucleolus but little electron dense heterochromatin is present. Identification of satellite cells is facilitated by their close apposition to muscle fibres and lack of intervening basement membrane. ×4400.

Summary of fibre and nuclear numbers in control and hypertrophic EDL muscles

Animal number	No. of fibres examined in sampling areas*		No. of nuclei counted		N_A (mm^{-2})		N_V ($\times 10^3$ mm^{-3})	
	Control	Hypertrophic	Control	Hypertrophic	Control	Hypertrophic	Control	Hypertrophic
1	178	177	135	117	396	343	21.64	18.74
2	216	190	159	146	467	428	25.52	23.39
3	243	188	170	131	499	384	27.27	20.98
4	218	175	165	130	484	381	26.45	20.82
5	170	158	125	126	367	370	20.05	20.22
6	204	162	131	122	384	358	20.98	19.56
7	214	142	160	116	469	340	25.63	18.58
8	188	208	165	158	484	464	26.45	25.36
	$\sum = 1631$	$\sum = 1400$	$\sum = 1210$	$\sum = 1046$	$\bar{N}_A = 443.8$	$\bar{N}_A = 383.5$	$\bar{N}_V = 24.25$	$\bar{N}_V = 20.96$

* In each animal, 18 randomly distributed non-overlapping microscopical sampling areas were used in both control and hypertrophic muscles for fibre and nuclear counting. Each sampling area was 4.26×10^{-2} mm^2 in area. $\bar{L}$ was estimated to be 18.3×10^{-3} mm by measuring the lengths of ~ 100 randomly selected nuclei seen in longitudinal muscle sections. The mean cross sectional areas of control and hypertrophic muscle fibres were found to be 441 μm^2 and 256 μm^2 respectively.

mature muscle fibres as satellite cells were seen at 18 days in both the electron microscopical (figure 2) and the succinate dehydrogenase preparations (figure 3). No satellite cell activity and satellite structures were found at 68, 98 and 128 days post-operatively. It is hard to escape the conclusion that increased satellite cell activity is related to the occurrence of the more mature fibre-like satellite structures.

Semithin (~ 1 μm) transverse and longitudinal sections were stained with toluidine blue[16,17] and used exclusively for stereological analysis of the myonuclear frequency (number of myonuclei per unit volume of muscle tissue, N_V) in ipsilateral hypertrophic and contralateral control EDL muscles. The number of myonuclei in ~ 300 randomly selected microscopical fields (× 220) were counted in transverse section and from knowledge of their number per unit area (N_A). N_V was obtained from the equation $N_V = N_A/\bar{L}$ where $\bar{L}$ is the mean length of 100 randomly selected nuclei seen in longitudinal sections[18]. The mean muscle fibre cross sectional area in each microscopical field was also measured. The result are expressed in the table and show that the overall mean reduction in N_V in hypertrophic muscles (to 85.8% of control value) is approximately equivalent to the increase in mean muscle fibre cross sectional area (~ 18%). This would seem to indicate that there is neither a significant loss of nuclei from muscle fibres nor a significant nuclear recruitment into fibres following the surgically induced hypertrophy.

12 animals at 14 and 28 days post-operatively were used for autoradiographic analyses to see whether nuclear recruitment from satellite cells to muscle fibres had occurred. 1 μCi/g b.wt of ^{3}H-thymidine was injected into each mouse and autoradiographs of ipsilateral and contralateral muscles were prepared using an emulsion dipping technique[19]. This was carried out in the expectation that labelled myonuclei would be found. This would have indicated that satellite cells had undergone division with subsequent fusion with parent fibres. Labelled myonuclei were not seen in either contralateral or ipsilateral EDL muscles though appreciable labelling of the increased amounts of

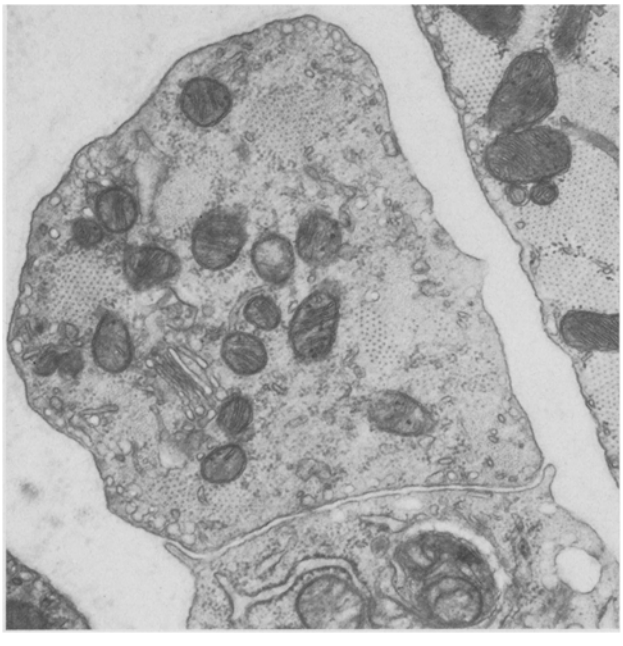

Fig. 2. Electron micrograph of satellite structure in 18-day hypertrophic EDL muscle. The sarcoplasm contains numerous mitochondria and Golgi apparatus and myosin filaments in hexagonal patterns in fibrils characteristic of skeletal muscle fibres. × 28,000.

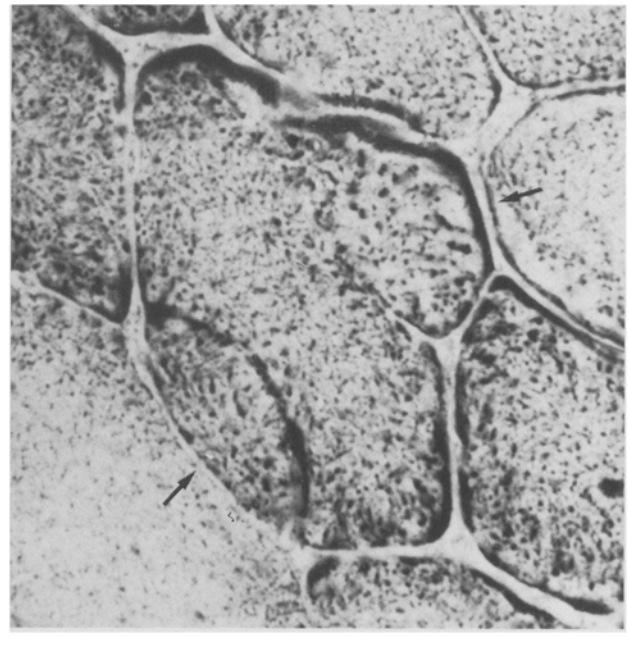

Fig. 3. Thick (~ 20 μm) transverse cryostat section stained for succinate dehydrogenase activity from 18-day hypertrophic EDL muscle. 2 satellite structures (arrowed) are clearly visible adjacent to a mature type I muscle fibre. × 520.

endomysial and perimysial connective tissue between fibres and fasciculi was noted in the ipsilateral EDL as previously reported by other investigators for the hypertrophic soleus muscle[20].

The finding that there was neither significant loss nor recruitment of myonuclei into hypertrophied muscle appears to suggest that true splitting of muscle fibres does not occur. If fibre splitting had actually occurred, nuclei of muscle fibres would have been lost to satellite structures or the smaller 'split' fibres, and then the fall in hypertrophic N_V values would have been much greater. The electron microscopic findings suggest that satellite structures take origin from satellite cells rather than by any splitting from muscle fibres. The failure to find ^3H tritium labelled nuclei in hypertrophic fibres would also seem to suggest that either fusion of satellite cell products with muscle fibres does not take place or that if it does occur extensive mitosis within satellite structures does not occur prior to fusion. Refusion of split fibres with their parent fibres or fusion of satellite structures with mature fibres would seem to be prohibited by the occurrence of a prominent endomysial sheath clearly demonstrable using picro sirius blue GL[12].

These findings lead us to believe that the transient histological features of hypertrophying muscles could be regarded as a reaction to the hypertrophy-inducing process rather than an essential feature of the mechanism of fibre size increase. The findings lead us to believe that split fibres or satellite structures either arise by satellite cell division but subsequently undergo complete regression or that they arise from satellite cell activity without significant mitosis and subsequently fuse with the previously existing fibres. These conclusions are at variance with the traditionally accepted views and remain to be tested experimentally.

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An improved method for calculating colony forming ability in soft agar with special reference to malignancy

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Summary. We examined the correlation between the tumorigenicity and the growth capacity in soft agar of various malignant cell lines. The colony forming rates were calculated in (a) 0%, (b) 0.15%, (c) 0.30%, and (d) 0.40% soft agar medium. An approximate value for the colony forming capacity, 'y', was then established for the formula $y=\frac{1}{a}\int_0^a f(x)\,dx$ (x = concentration of soft agar, f(x) = rate of appearance of colony forming cells with x% soft agar medium).

Malignancy in cultured cell lines is indicated by the properties the cell lines show in vitro or in vivo such as a) loss of contact inhibition[2], b) growth in soft agar[3], c) growth in spinner culture[4], and d) tumorigenicity[5]. The most reliable method of determining the degree of malignancy would be to examine the tumorigenicity of each cultured cell line, but it is not possible in a short-term experiment to do a quantitative study. In order to study the mechanism by which malignancy is controlled, a quantitative study of the cell variation seen in malignant cells is needed. In this experiment we introduced a new method of calculating the colony forming ability, and investigated whether or not cell growth in soft agar correlates with tumorigenicity, and whether or not the former could be applied to the quantitative study of malignancy in vitro.

Materials and methods. The cell lines used were Ht(A/Jax mouse), F(A/Jax), MC(A/Jax), m(A/Jax), L(C_3H), MRCB(A/Jax), SY(A/Jax), and ME(A/Jax) as well as normal mouse embryonic cells (MEb) and normal human embryonic lung cells (HEL).

Tumorigenicity: The tumorigenicity of the various cell lines which we used was tested by injecting 1×10^6 cells into the subcutaneum of an isologous strain of newborn, adult and nude mice(BALB/c, nu/nu). The latency period (the length of time until tumors were palpable in the site at which the cells were injected) and the days of survival of 50% of the mice into which the cells were injected, were noted.

Cell growth in soft agar: 5000 cells were seeded in a petri dish and their colony forming ability was examined on the 5th day after cell seeding. Any group of cells consisting of more than 6 cells derived from a single cells was classified as a colony. We ascertained that the cell lines which we used, except for normal cell (MEb and HEL), ME and SY cell lines, by the 5th day had produced colonies large enough to be identified under the microscope, and that the colony forming cells had divided an average of 2.5 times within a period of 5 days. Normal cell (MEb and HEL), ME and SY cell lines were observed for about 1 month in order to determine whether or not they had colony forming ability. For each group of 3 dishes, the mean value of the numbers of colonies found in them was calculated and the colony forming rates were obtained.

Preparation of bottom agar layer: 6 ml of 0.6% agar growth medium were poured into 60-mm petri dishes and allowed