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INNERVATION OF FREE MUSCLE GRAFTS IN THE RAT IN THE ABSENCE OF MECHANICAL TRAUMA TO SURROUNDING MUSCLES OR NERVES

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Summary

Soleus muscles in the rat were freely grafted alongside a normal soleus muscle in the absence of mechanical trauma to any of the surrounding muscles or motor nerves. The object of this experiment was to determine whether or not the muscle grafts would become reinnervated under these circumstances. Contractile and histochemical properties of the grafts were compared with those of the contralateral denervated soleus as well as normal muscles. Innervation of the grafts did occur, and it was concluded that the innervation of the grafts arose primarily from sprouts from nerves supplying neighboring muscles. The grafts were studied with specific nerve stains, histochemical techniques and by analysis of their contractile properties.

Studies on the free autografting of rat muscles have demonstrated the reinnervation of the grafts by fibers regenerating from the end of the transected nerve lying in the bed of the graft (2,3,6). Even though the cut end of the motor nerve is not fixed to the muscle, spontaneous reinnervation consistently occurs. In clinical practice, free muscle grafting is often used to supplant facial muscles which have atrophied because of unilateral facial paralysis. Reinnervation of grafted muscles in humans may also be accomplished without the direct suturing of nerve stumps to the grafts. Commonly the belly of the muscle graft is placed into the innervated side of the face, with a tendon extending to an insertion point on the paralyzed side. The graft is then innervated by nerve sprouts arising from underlying normal muscles (14). To facilitate reinnervation, the surgeons have empirically found it useful to scrape off the fascia and to damage opposing surfaces of both the graft and the muscle which is to supply the motor nerves. Scraping is assumed to eliminate a mechanical barrier to the ingrowth of nerve fibers into the graft.

Age does not appear to be a major factor in determining whether or not a free muscle graft will become innervated. Free muscle grafts have been successfully performed in patients ranging from young adulthood to moderate old age. In addition, muscle grafts in old (26 month) rats become reinnervated (7).

Most experiments on the free grafting of muscles in the rat have involved the orthotopic grafting of limb muscles in young animals. In orthotopic free grafting, a muscle is completely removed from the body and is then replaced, with normal orientation, into its own bed. The present experiments were designed to develop an approach to studying the mechanism of reinnervation of muscle grafts, particularly for situations in which no motor nerves have been transected. The major question was whether or not an untraumatized muscle, grafted into a limb without direct trauma to neighboring muscles or nerves, would become reinnervated by a process of collateral regeneration from nerves in neighboring (but not traumatized) muscles.

Materials and Methods

These experiments were conducted upon 50 one-month old male rats of the Wistar (60 generation inbred) and Sprague-Dawley strains. Surgery itself was performed with the animals under ether anesthesia. A skin incision was made over the soleus muscle, and by blunt dissection, a space was created in the loose fascia alongside the soleus muscle. The fundamental strategy was to insert a free soleus graft into the fascial space without causing any direct damage to either the muscles or motor nerves surrounding the bed of the graft. Two varieties of the operation were performed. In Series I, the left soleus of fifteen 60 gm Sprague-Dawley rats was removed and grafted into the fascial space between the right soleus muscle and the peroneal muscle group. Insertion of the graft into this space can be performed without any damage to the surrounding muscles. In Series II, a soleus muscle from a 30 day inbred Wistar rat was ipsilaterally grafted into a space created between the soleus and gastrocnemius muscle of a recipient of the same size and sex. The graft (if not reinnervated by collateral regeneration) would show the properties of a denervated muscle. As a control for contractile and histochemical studies, the contralateral soleus of the recipient was denervated by section and ligature of the sciatic nerve. All grafted muscles were sutured to the Achilles tendon distally and near the origin of the soleus proximally to maintain proper resting length and a functional environment.

Contractile analysis. In 26 cases, muscles (the soleus graft, the normal soleus alongside the graft and the contralateral denervated soleus) were removed from rats under ether anesthesia. The muscles were placed in an oxygenated tissue culture medium, to which tubocurarine had been added. After the resting length of the muscle was set to the tension eliciting maximal twitch tension, the following contractile properties were measured with an automatic analyzer (1,2): twitch tension, latency period (LP - the time from stimulation to first mechanical response), contraction time (CT - time to peak), and half relaxation time (HRT).

Histological and histochemical analysis. The grafts that were subjected to contractile analysis were frozen in liquid nitrogen and analyzed histochemically for succinic dehydrogenase

[SDH] (9), myosin adenosine triphosphatase [ATPase] (5,10), and phosphorylase (13) activity. With these stains the muscle fibers could be broken down into fast twitch glycolytic, fast twitch oxidative-glycolytic and slow twitch oxidative categories corresponding to those proposed by Peter et al. (12). Other grafts were fixed in Bouin's, sectioned and stained with hematoxylin and eosin or Palmgren's silver stain for nerve fibers. In addition, frozen sections were stained for motor end plates with Henderson's modification of Gomori's acetylcholinesterase method (11).

Results

Series I. Implantation of free graft between soleus and peroneal muscles. Initially the grafts did not differ in their reaction to transplantation from orthotopic soleus grafts (3). After the third week, the grafts became progressively thinner and were represented by thin bundles of muscle fibers associated with bands of connective tissue. In all of the grafts except one, however, nerves were found among the regenerated muscle fibers. Despite the fact that reinnervation of grafts placed between the soleus and peroneal muscles did occur, this grafting site was abandoned in favor of placing the grafts between the soleus and gastrocnemius muscles. This was done because the condition of the grafts in the latter site was better, making them more suitable for the analysis of contractile properties.

Series II. Implantations of free grafts between soleus and gastrocnemius muscles. Five experimental groups involving 26 animals were subjected to contractile and histochemical analysis. The contractile data from only three groups will be presented in full (Table 1). The overall design of the fourth group was identical to that of the groups reported in Table 1, except that the animals were 2 1/2 months instead of one month old at the time of grafting. In the fifth group, the soleus muscle next to the graft was denervated and the contralateral soleus was normally innervated. The results of these latter two groups were comparaable with those of the three groups reported in Table 1. Ten grafts, treated like those in groups 1-3 were analyzed for the presence of nerve fibers and motor end plates.

The long term grafts in Series II contained a larger proportion and absolute mass of muscle fibers than the grafts placed between the soleus and peroneal muscles (Series I). In all of these groups, there was evidence of motor innervation of the grafts, but the variables inherent in the experiment made it necessary to use several different techniques in order to interpret properly the nature of the innervation of the grafts. The contraction times of the soleus grafts (Table 1) are similar to those of non-transplanted soleus muscles which have been denervated for a period of time equal to the age of the graft. They are, however, also similar to those of the lateral head of the normal gastrocnemius muscle. The twitch tensions of the free grafts show a mean value of 1.40 gm at 60 days whereas in the 60 day denervated, but not transplanted, soleus the mean twitch tension is only 0.10 gm. The variability of the twitch tensions of the grafts (0.99 gm at 14 days, 0.55 gm at 30 days and 1.40 gm at 60 days) is not atypical for soleus grafts, and it is a reflection of their functional mass. For unknown reasons more

TABLE 1. Contractile Properties of Duplicate Soleus Grafts Compared with Normal and Denervated Soleus Muscles

Experimental Group[1]	Twitch Tension (gm)	Latency Period (msec)	Contraction Time (msec)	Half Relaxation Time (msec)	Muscle Wt. (mg)	Wt. of Rat (gm)
14 Days (n=4)						
Free Soleus Graft	0.99 ±0.04	3.85 ±0.33	28.45 ±0.43	26.40 ±0.43	18.0 ±2.74	93.80 ±3.15
Normal Soleus (next to graft)	2.89 ±0.88	3.30 ±0.08	30.34 ±1.23	37.70 ±1.89	40.0 ±4.34	"
Contralateral Denervated Soleus	0.27 ±0.06	4.35 ±0.23	28.00 ±0.55	34.50 ±1.62	19.5 ±2.25	"
30 Days (n=6)						
Free Soleus Graft	0.55 ±0.20	3.86 ±0.23	26.66 ±2.39	30.74 ±2.92	22.0 ±4.01	202 ±10.56
Normal Soleus (next to graft)	4.71 ±1.22	3.28 ±0.05	32.62 ±1.35	40.27 ±3.45	83.3 ±10.62	"
Contralateral Denervated Soleus	0.31 ±0.04	3.94 ±0.29	28.42 ±1.95	34.22 ±4.91	22.4 ±1.94	"
60 Days (n=4)						
Free Soleus Graft	1.40 ±0.57	3.60 ±0.48	18.00 ±3.28	20.40 ±2.25	39.2 ±6.02	261 ±17.49
Normal Soleus (next to graft)	18.55 ±1.35	3.38 ±0.11	34.52 ±2.06	52.52 ±4.51	126.2 ±6.24	"
Contralateral Denervated Soleus	0.10 ±0.04	4.43 ±0.14	20.25 ±1.12	27.45 ±2.47	13.5 ±2.33	"
Normal gastrocnemius of 60 day rat (from 1)	--	3.5	17.8	12.8	--	--
30 day minced gastrocnemius regenerate (from 1)	--	4.9	21.9	30.7	--	--

[1] For each day of analysis, three soleus muscles from the same rat were analyzed. The free soleus graft, taken from an inbred donor, was placed between the soleus and gastrocnemius muscles in one leg. The normal soleus is the undamaged, normally innervated soleus muscle alongside which the graft was placed. The contralateral denervated soleus was taken from the opposite leg of the same rat.

variability is seen in the functional mass of soleus grafts than in grafts of other muscles. Overall, the contraction times of the soleus muscles in these experiments are somewhat shorter than those reported in adult rats because in normal development the soleus undergoes a temporary phase of speeding during the period covered by these experiments (8).

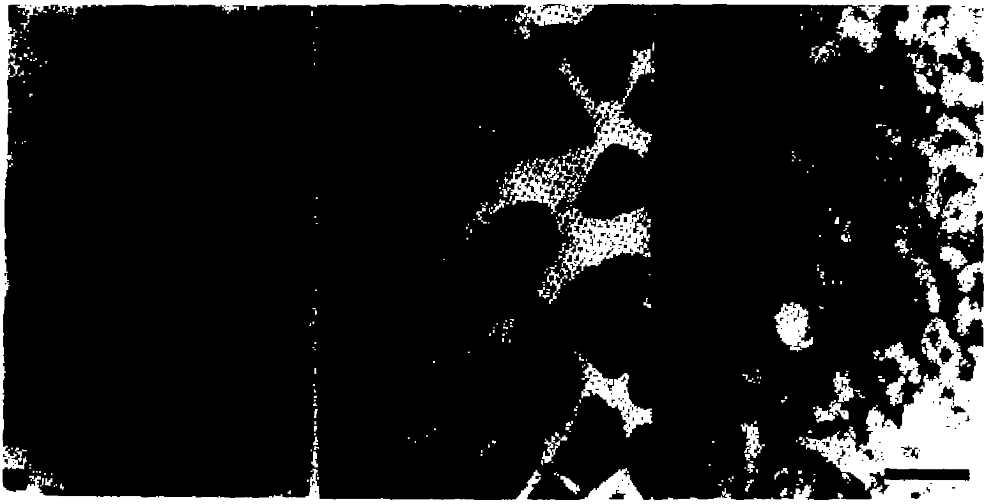

FIG. 1

a. Normal soleus muscle located alongside the graft shown in Fig. 1,b. ATPase. The muscles in parts a, b, and c are from the same rat and are shown at the same magnifica- ation. Calibration bar (in c) = 50 μm.

b. Sixty day soleus graft stained for ATPase activity. The diameter of the fibers is large. Light and dark fibers are bunched into groups.

c. Soleus muscle from contralateral leg. The muscle was denervated 60 days previously. Note the greatly reduced diameters of the muscle fibers. ATPase.

The histochemical preparations (SDH, ATPase and phosphorylase) made from the grafts revealed large muscle fibers and the differentiation of histochemical muscle fiber types in the grafts (Fig. 1,b). The grafts (Fig. 1,b) also demonstrated type grouping of the muscle fibers (indicative of the reinnervation process) instead of the normal checkerboard pattern (Fig. 1,a). This picture contrasted to the advanced atrophy and decrease of enzyme activity of the denervated muscle (Fig. 1,c).

The histological and histochemical evidence provided both direct and indirect evidence of innervation of the grafts. Silver stained paraffin sections routinely demonstrated the presence of nerve fibers among the muscle fibers of the grafts (Fig. 2), and motor end plates were demonstrated histochemically (Fig. 3).

FIG. 2
Thirty two day soleus graft demonstrating abundant nerve fibers (arrow) among the muscle fibers. Palmgren silver stain. Calibration bar = 150 μm.

FIG. 3
Motor end plate present in a 38 day soleus graft. Acetylcholinesterase stain. Calibration bar = 50 μm.

Discussion

The main finding from these experiments is that reinnervation of a freely grafted soleus muscle does occur in the absence of direct trauma to the graft, to the surrounding muscles or to the motor nerves in the vicinity of the graft. In contrast, reinnervation does not occur in a locally denervated, but otherwise untraumatized, rat muscle if suitable precautions (ligature of the stump and embedding it into another muscle) are made.

This is clearly shown by the progressive atrophy and ultimate replacement of the denervated muscle by connective tissue and fat. The chief differences between a free soleus graft, as reported in this paper, and the denervated, intact soleus is that the graft, being avascular during the first few postoperative days, undergoes a massive breakdown of its muscle fibers, followed in turn by the massive regeneration of new muscle fibers that have never been exposed to nerves. It is likely that in some manner this condition is communicated to intact nerves surrounding the graft. Whether there is a positive ("neurotropic") stimulus, emanating from the graft to the neighboring nerves or, instead, the absence of negative feedback, is not known at this time. It is possible that both may be operative at different phases of the reinnervation process.

Analysis of reinnervation of the graft is complex because several patterns of reinnervation are possible. The main options are 1) no reinnervation, 2) reinnervation from the intact soleus nerve, 3) reinnervation from nerves to other neighboring muscles (all fast), and 4) some combination of the above.

The fact that the grafts were innervated was established by 1) the demonstration of nerve fibers and motor end plates in them, 2) the marked difference in fiber size and pattern (though not in all cases) compared with that of the denervated soleus muscle, 3) the presence of different histochemical fiber types, 4) the grouping of fiber types, and 5) the twitch tensions of the grafts as compared with those of denervated soleus muscles. From the contraction times alone, it would not be possible to tell whether the grafts had remained denervated or whether they had been innervated by a nerve other than the soleus nerve. This is due to the shortening of the contraction time occurring in the soleus muscle of animals denervated at the age of one month. Thus, analysis of contraction times was the least useful technique in demonstrating whether or not the graft was innervated.

It is known that orthotopically grafted muscles are spontaneously reinnervated by their own motor nerves and that in both fast and slow muscles the contraction times approach closely those of the normal muscle (2). It is also known that in cross-transplanted muscles the contraction times of the grafts are converted to correspond with the properties of the nerve that innervates the graft. In the cross-transplanted rat soleus, the conversion of contraction times towards those of a fast muscle is almost complete (6). With the knowledge that the nerve grafts were innervated, the contraction times allow one to conclude that the source of the innervation was most likely sprouting from nerves from a nearby fast muscle. The main evidence is, however, provided by the histochemical findings. At this point, a minor contribution from the soleus nerve cannot be ruled out, but if there was one, it was not enough to exert a significant effect upon the contractile properties.

In clinical application of free muscle grafting, particularly in cases of unilateral paralysis of the face, the muscle graft is placed over a muscle on the innervated half of the face, and the opposing edges of the graft and the normal underlying muscles are scraped free of fascia and otherwise traumatized in order to facilitate the ingrowth of nerves into the graft (14). In an experimental study on rats (4), free muscle grafts placed over the intercostal region, apparently after cutting the cutaneous maximus muscle, were found to become innervated. The present study demonstrates that reinnervation of free muscle grafts can occur in the absence of major trauma to any of the muscles or nerves in the vicinity of the graft. Further work must be done to learn how to control the sites from which the collateral sprouting from the intact nerves will occur.

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References

1. B. M. CARLSON and E. GUTMANN, Exp. Neurol. 36 239-249 (1972).
2. B. M. CARLSON and E. GUTMANN, Pflügers Arch. 353 215-225 (1975a).
3. B. M. CARLSON and E. GUTMANN, Anat. Rec. 183 47-62 (1975b).
4. H. GRUBER, G. FREILINGER, J. HOLLE and R. MAYR, Experientia 30 1191-1192 (1974).
5. L. GUTH and F. J. SAMAHA, Exp. Neurol. 28 365-367 (1970).
6. E. GUTMANN and B. M. CARLSON, Pflügers Arch. 353 227-239 (1975).
7. E. GUTMANN and B. M. CARLSON, Life Sci. 18 109-114 (1976).
8. E. GUTMANN, J. MELICHNA and I. SYROVY, Physiol. Bohemoslov. 23 19-27 (1974).
9. M. M. NACHLAS, K. C. TSOU, E. SOUZA, C. S. CHENG and A. M. SELIGMAN, J. Histochem., Cytochem. 5 420-436 (1957).
10. H. A. PADYKULA and E. HERMAN, J. Histochem. Cytochem. 3 170-195 (1955).
11. A. G. E. PEARSE, Histochemistry, Vol. 2, 3rd ed., p. 1312. Church: 11 Livingstone, London (1972).
12. J. B. PETER, R. J. BARNARD, V. R. EDGERTON, C. A. GILLESPIE and K. E. STEMPEL, Biochemistry 11 2627-2633.
13. T. TAKEUCHI and H. KURIAK, J. Histochem. Cytochem. 4 153-161 (1955).
14. N. THOMPSON, Clin. Plastic Surg. 1 349-403 (1974).