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Endothelial cell activation during angiogenesis in freely transplanted skeletal muscles in mice and its relationship to the onset of myogenesis

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INTRODUCTION

The regeneration of skeletal muscle following injury is dependent upon an adequate nutrient supply to the damaged area (Carlson & Faulkner, 1983). This becomes particularly important in the case of freely transplanted skeletal muscles where the transplant is completely avascular, and nutrients have to be derived from adjacent tissues (Hansen-Smith, Carlson & Irwin, 1980).

In transplanted muscles the intrinsic degeneration of myofibres that precedes regeneration (Carlson & Gutmann, 1975; Hansen-Smith & Carlson, 1979) is accompanied by a parallel degeneration of blood vessels within the transplant (Hansen-Smith *et al.* 1980). Such degeneration is characterised by an almost complete disintegration of the endothelium and, in larger vessels, a degeneration of the smooth muscle cells of the tunica media. Only the basal laminae remain intact (Hansen-Smith *et al.* 1980).

For the transplant to regenerate successfully, it is necessary that a vascular supply be rapidly re-established, firstly to allow phagocytes, which ingest debris from the intrinsic degeneration of myofibres, to enter the transplanted muscle and, secondly, to provide a functional nutrient supply for muscle regeneration (Hansen-Smith & Carlson, 1979; Carlson & Faulkner, 1983).

Reintroduction of a vascular supply may begin with the outgrowth of surviving blood vessels from the periphery of the transplant towards functioning vessels in the surrounding tissues (Faulkner, Weiss & McGeachie, 1983). Of greater significance however is the growth, from surrounding tissues, of endothelial-lined tubes which eventually make contact with basal laminae in the transplant. Newly formed endothelial cells seemingly use the basal laminae as guides for growth into the transplant. The new vessels formed within these basal laminae, like the newly formed capillaries, arterioles and venules, show structural abnormalities (Hansen-Smith *et al.* 1980).

In this study we use autoradiographic techniques to investigate the activation of endothelial cells in intact transplants of the extensor digitorum longus muscles (EDL) and in tibialis anterior muscles (TA) over which the EDL muscles were transplanted in mice. All neural and vascular connections of the transplants were severed.

This experiment also enabled a comparison to be made with the activation time of myogenic precursor cells, as reported in a previous paper (Roberts, McGeachie, Grounds & Smith, 1989), by allowing the observation of pre-mitotically labelled (presumptive) satellite cells prior to regeneration, compared with post-mitotically

labelled myotube nuclei, which were identified after regeneration had occurred some 14 days after transplantation.

MATERIALS AND METHODS

Animals and surgical procedures

The animals used were 26 young (6–8 weeks) mature male BALB/c mice (20–25 g). Mice were anaesthetised with diethyl ether throughout the transplantation.

The bilateral transplantation procedure involved locating and severing the distal tendon of the EDL and, with the proximal tendon still intact, bluntly dissecting the muscle from its bed. The proximal tendon was tied with a loop of 7/0 braided silk suture (Ethicon), divided, and immediately sutured to the distal tendon of the quadriceps femoris muscle of the same leg. The EDL was relocated longitudinally over the TA muscle, and was extended to its normal (pre-excision) length before a loop of 7/0 braided silk suture was tied around the distal EDL tendon and anchored to the distal tendon of the TA. All neurovascular connections to the transplanted EDL were severed during the procedure. A total of 52 autotransplants were inserted in 26 mice.

Autoradiography and sample analysis

Tritiated thymidine (6-3H-thymidine, 3H-TdR, specific activity 5·0 Ci/mmol, Amersham International, UK) at a dosage of 1 μCi/g of body weight was injected intraperitoneally into each mouse. Only one injection of 3H-TdR was given to each mouse. Two mice were injected at each of the following times after transplantation: 11, 23, 35, 47, 59, 95, 119, 143, 167, 191 and 336 hours. Four mice were injected at 71 hours. Tritiated thymidine is available for about 1 hour after injection and is incorporated into the nuclei of all cells synthesising DNA during this period.

One hour after injection, the pairs of mice were given a lethal dose of sodium pentobarbitone (80 mg/kg) before being perfused (through the heart, at a constant pressure) with 20 ml of heparinised saline, followed by 20 ml of 0·1 M phosphate-buffered 10% formal saline solution at pH 7·2. After perfusion, the transplanted EDL and part of the TA muscle were removed, cut transversely in half, and immersed overnight in full strength fixative at 4 °C.

Tissues were postfixed in 1% osmium tetroxide for 1 hour, washed in 0·1 M phosphate buffer, and block stained in 1% paraphenylenediamine in 70% ethanol for 1 hour to obviate tissue staining after autoradiography (Dilley & McGeachie, 1983). Tissues were infiltrated and embedded in Araldite, before 1 μm transverse sections were cut from the central area of each transplant (midway between the proximal and distal tendons), placed on glass slides, and coated with Kodak AR10 autoradiographic stripping film.

The slides were placed in light-tight boxes and exposed for 10 weeks at −20 °C, before being developed in Kodak D19, fixed in acid hardener-fixer, washed and dried.

Autoradiographs were examined histologically, before being analysed for labelled endothelial cell nuclei and muscle nuclei (myonuclei and presumptive satellite cell nuclei). Only labelled nuclei observed within the contour of a muscle fibre were considered to be presumptive satellite cell nuclei and not myonuclei; the latter are quiescent and incapable of DNA synthesis (Bischoff & Holtzer, 1969). A light microscope with a × 100 oil immersion lens was used for the analysis. In both the EDL and TA muscles 11873 endothelial cell nuclei and 10634 muscle nuclei were counted (totalling 22507 nuclei). A control group to ensure that only cells actively synthesising DNA would incorporate 3H-TdR was not used as the normal level of cell turnover is

very small. This was demonstrated in a related experiment (McGeachie, 1989) where intact muscles in mice were injected with 3H-TdR daily for seven days. Only $0{\cdot}14 \pm 0{\cdot}08\%$ of muscle (satellite cell) nuclei and $0{\cdot}28 \pm 0{\cdot}07\%$ of connective tissue (including endothelial cell) nuclei incorporated the label.

RESULTS

Histology

Vascular system

Judging by the presence of densely packed erythrocytes within blood vessels of the transplanted EDL (Fig. 1), no functioning blood supply was re-established within 60 hours of transplantation. If vascular connections had been established between the transplant and TA muscle the perfusion would have cleared these vessels, as was the case for blood vessels in the underlying TA. By 72 hours, (when 8 transplants were examined) only one transplant showed peripheral revascularisation penetrating to a depth of 120–150 μm at the surface of the transplant/TA interface. Even after the next time interval (at 96 hours) only 2 of the 4 transplants showed peripheral revascularisation, these both coming from the same animal. At 120 hours post-transplantation all muscles showed peripheral revascularisation which encompassed the entire periphery to a depth of 120–150 μm, with blood vessels of the central areas still being densely packed with erythrocytes. By 144 hours the transplants were almost completely revascularised, with only very small central areas containing blood vessels with densely packed erythrocytes in their lumens.

The first evidence of cellular infiltration into the transplant was seen in samples removed 72 hours after transplantation; this was evidenced by occasional migration of macrophages into the periphery of the transplants.

Endothelial cell nuclei in the larger blood vessels and in some of the smaller vessels of the transplants were swollen, although not pyknotic, at all periods between 12 and 96 hours after transplantation. Pyknotic nuclei were first evident in transplants removed 120 hours after insertion.

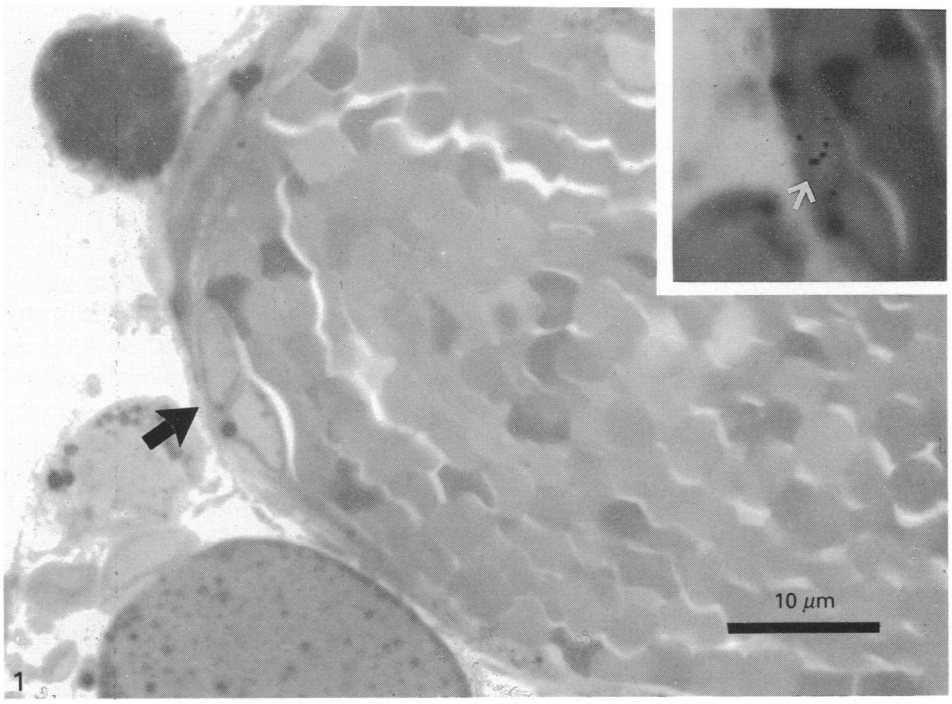
Following revascularisation (between 72 and 120 hours), observation of the blood vessels in the transplants revealed that although capillaries appeared to have a normal morphology, many of the larger blood vessels were irregularly shaped and abnormally large.

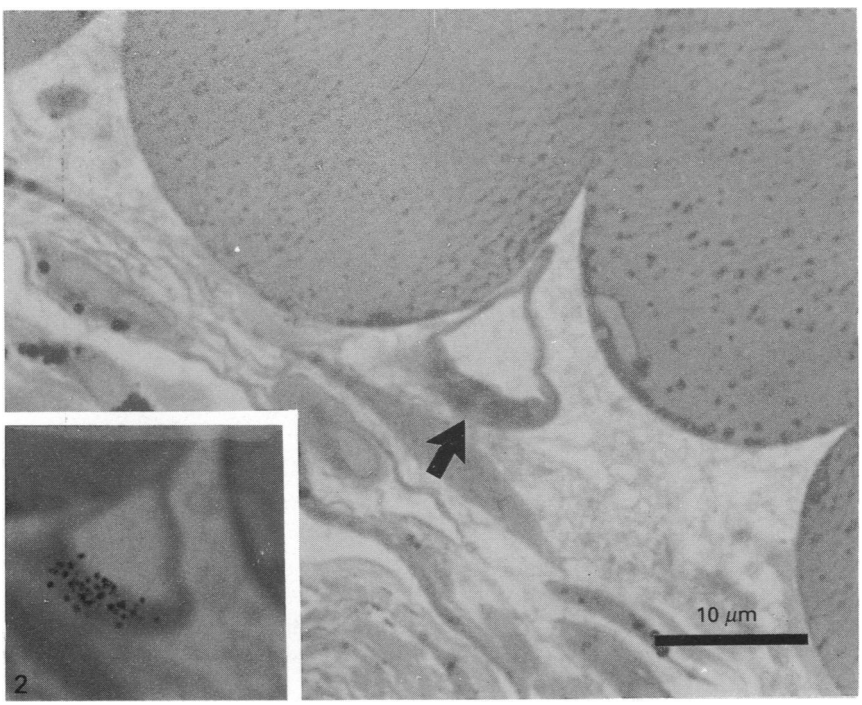
Muscle fibres

Twelve hours after transplantation, myofibres within the transplant were beginning to degenerate; myonuclei were difficult to distinguish and the myofibres were pale staining. By 24 hours various degrees of muscle degeneration could be seen within the transplants. In peripheral regions some fibres had survived and appeared to be unaffected, while others showed marked degeneration. By contrast, no signs of obvious disintegration were seen in fibres of the central region, although in this area the nuclei of myofibres were not discernible. The area intermediate between the centre and periphery of the transplant was marked by fibre autolysis and degeneration.

From 24 to 60 hours after transplantation there was little discernible difference in the appearance of the transplants. By 60 hours there was still no evidence of an inflammatory response. Myofibres, although pale staining on the whole, retained their shape, and there was no evidence of macrophage infiltration, though autolysis of some myofibres was well advanced.

Seventy two hours after transplantation, the peripheral zone (2–3 fibres or 150 μm


10 μm
1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2

Table 1. *The numbers of endothelial cell nuclei counted and labelling indices in EDL transplants and underlying TA muscles*

Time of 3H-TdR injection (hr)	No. of endothelial cell nuclei counted		Number labelled	Percentage labelled
11*	EDL	291	0	0
	TA	—	—	—
23	EDL	185	0	0
	TA	103	0	0
35	EDL	259	1	0·4
	TA	272	22	8·1
47	EDL	341	0	0
	TA	145	10	6·9
59	EDL	332	0	0
	TA	356	21	5·9
71†	EDL	619	6	0·97
	TA	745	43	5·77
95	EDL	630	42	6·67
	TA	448	38	8·48
119	EDL	1471	117	7·95
	TA	433	19	4·39
143	EDL	881	92	10·44
	TA	438	21	4·79
167	EDL	1068	38	3·56
	TA	382	1	0·26
191	EDL	1142	97	8·5
	TA	527	3	0·56
335	EDL	645	2	0·31
	TA	160	1	0·62

Four EDL transplants were examined at each time interval. Tissues were removed one hour after injection of 3H-TdR. EDL, EDL transplant; TA, underlying TA muscle.

* At the 11 hour interval, TA muscles were rendered unusable following faulty tissue processing.

† Eight transplants were examined at 71 hours.

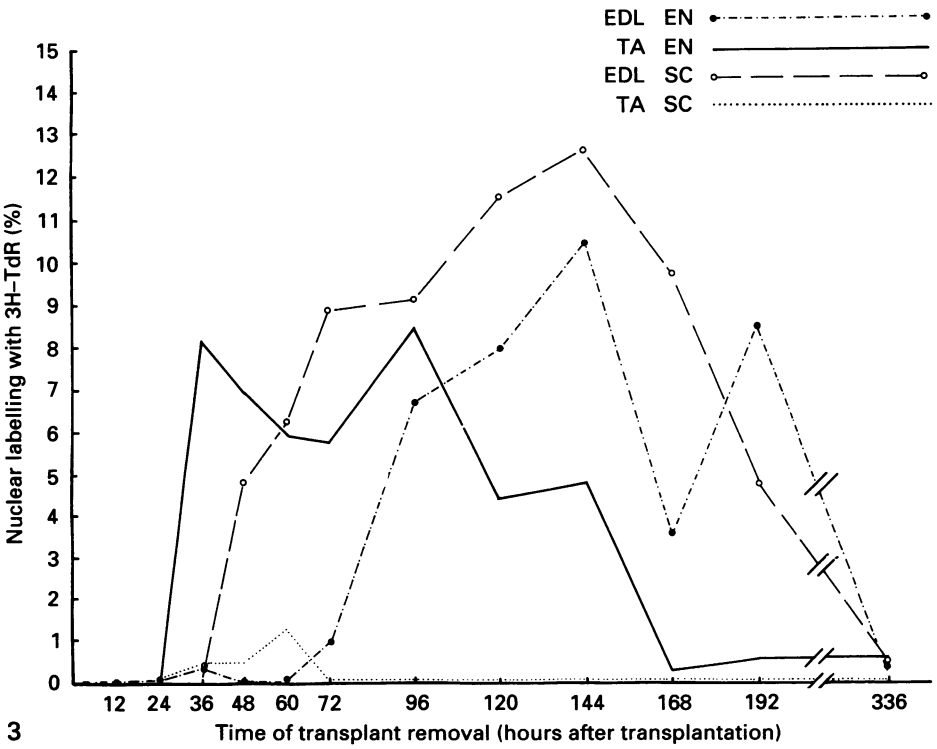
in depth) contained some surviving muscle which was interspersed with myofibres in various stages of degeneration. A number of cells (erythrocytes, fibroblasts and a few macrophages) were evident in the area between the transplant and TA muscle.

Myotubes were seen for the first time at 96 hours after transplantation, and pyknotic myonuclei were first seen at 120 hours. From this time onwards the degeneration and regeneration of the transplants show a radial pattern from the periphery to the centre, as previously described by Carlson & Faulkner (1983).

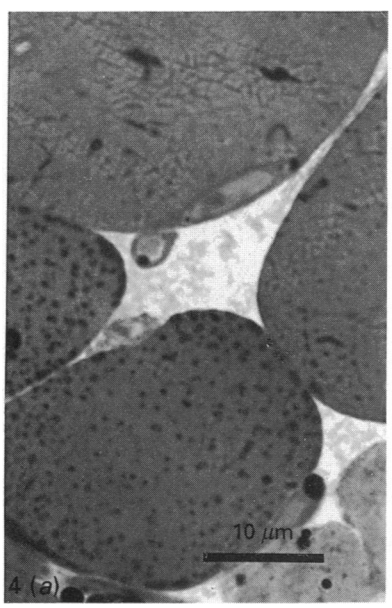
Even 8 days (192 hours) after transplantation there was still a central core of degenerating myofibres surrounded by small immature myotubes. Fourteen days after transplantation the EDL showed the classical appearance of a regenerated muscle transplant, with a rim of surviving muscle fibres surrounding a large central core of myotubes.

Fig. 1. A high power photomicrograph (transverse section) through a large blood vessel at the periphery of a transplanted EDL muscle removed 35 hours after transplantation. The blood vessel is densely packed with erythrocytes. Inset: The nucleus of an endothelial cell within the wall of the blood vessel (arrowed) showing autoradiographic grains in focus. The animal was injected with 3H-TdR one hour previously.

Fig. 2. A high power photomicrograph taken at the periphery of the TA muscle over which the EDL transplant was placed. The animal was injected with 3H-TdR 59 hours after transplantation and sampled one hour later. The main photograph shows a capillary (arrowed), surrounded by TA muscle fibres. Inset: Autoradiographic grains in focus over the endothelial cell nucleus of the same capillary.


EDL EN
TA EN
EDL SC
TA SC
Nuclear labelling with 3H-TdR (%)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
12 24 36 48 60 72 96 120 144 168 192 336
Time of transplant removal (hours after transplantation)

3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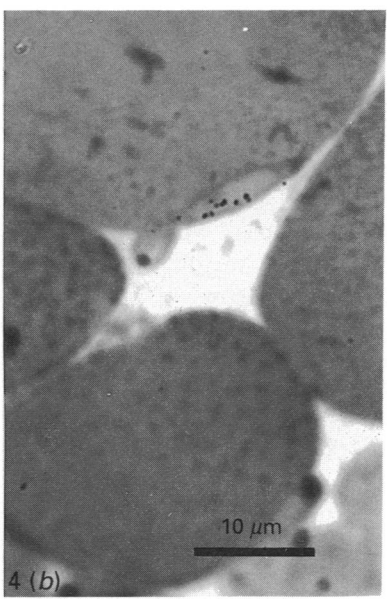
4 (*a*)


10 μm

4 (*b*)

Table 2. *The numbers of muscle nuclei (myonuclei and presumptive satellite cell nuclei) counted, plus their labelling indices*

Time of 3H-TdR injection (hr)	Number of muscle nuclei counted		Number labelled	Percentage labelled
11*	EDL	313	0	0
	TA	—	—	—
23	EDL	207	0	0
	TA	404	0	0
35	EDL	300	0	0
	TA	378	2	0·5
47	EDL	476	23	4·83
	TA	202	1	0·5
59	EDL	485	30	6·2
	TA	467	6	1·3
71†	EDL	792	70	8·84
	TA	746	1	0·13
95	EDL	397	36	9·06
	TA	420	0	0
119	EDL	685	79	11·53
	TA	463	0	0
143	EDL	681	86	12·62
	TA	473	0	0
167	EDL	565	55	9·73
	TA	550	1	0·18
191	EDL	509	24	4·72
	TA	569	0	0
335	EDL	205	1	0·48
	TA	347	0	0

Four EDL transplants were examined at each time interval. Tissues were removed one hour after injection of 3H-TdR. EDL, EDL transplant; TA, underlying TA muscle.

* At the 11 hour interval, the TA muscles were rendered unusable following faulty tissue processing.

† At 71 hours, 8 transplants were examined.

Autoradiography

The first evidence of nuclear labelling was in fibroblasts at the periphery of the transplants at 24 hours. They were also labelled in the area between the transplant and TA muscle, and within the periphery of the transplant up to 72 hours and at later times throughout the degeneration and regeneration.

Vascular system

The first evidence of endothelial cell activation within the 150 μm peripheral zone of the transplants was labelling in one endothelial cell nucleus in the wall of a large blood vessel (Fig. 1). This labelled nucleus was in a transplant injected with 3H-TdR at 35 hours after insertion and sampled one hour later. The vessel containing this labelled endothelial nucleus was at the very edge of the transplant and was morphologically similar to those reported by Faulkner *et al.* (1983). Its lumen was

Fig. 3. Graph showing the mean percentage labelling with 3H-TdR at all time intervals sampled. EDL EN, endothelial cell nuclei in EDL transplants; TA EN, endothelial cell nuclei in TA muscles underlying the EDL transplants; EDL SC, presumptive satellite cell nuclei in the EDL transplants; TA SC, presumptive satellite cell nuclei in TA muscles underlying the EDL transplants.

Fig. 4 (*a–b*). Muscle fibres at the periphery of a transplant removed 48 hours after insertion. (*a*) shows the muscle fibres in focus, whereas (*b*) shows autoradiographic grains in focus over a premitotic presumptive satellite cell nucleus. 3H-TdR was injected one hour prior to tissue sampling.

densely packed with erythrocytes, and the transplant did not appear to be revascularised. It was the only labelled endothelial cell seen in 20 transplants analysed up to 60 hours after transplantation. At 72 hours one transplant (of 8 sampled) contained 6 labelled endothelial cell nuclei. Even by 96 hours after transplantation only half of the 4 transplants sampled contained labelled endothelial cell nuclei. At 8 days (192 hours) after transplantation labelled endothelial cell nuclei were abundant, but at the last time interval of 14 days (336 hours) only an occasional labelled nucleus was seen. The numbers of endothelial cell nuclei counted and percentages labelled are shown in Table 1.

By contrast, labelled endothelial nuclei were plentiful within the 150 μm zone beneath the transplants in the TA muscle, from 36 to 120 hours after transplantation (Fig. 2), and even at 336 hours (14 days) the occasional labelled nucleus was seen (see Table 1 and Fig. 3). That is, at times whilst there was almost no activity in the transplant, endothelial cell nuclei of blood vessels in the adjacent TA were proliferating.

Muscle fibres

Labelled presumptive satellite cell nuclei were seen within both degenerating and surviving muscle fibres in the transplanted EDL muscles from 48 hours after transplantation, although only within the 150 μm peripheral zone (Fig. 4). From 36 to 72 hours post-transplantation labelled presumptive satellite cells were also seen in the TA muscle underlying the transplant. The numbers of muscle nuclei counted and percentages labelled (in both the transplant and TA) are shown in Table 2 and Figure 3.

DISCUSSION

The aim of the present study was to establish the initiation and timing of endothelial cell proliferation in small intact EDL skeletal muscle transplants by detecting the premitotic incorporation of 3H-TdR. The study was also designed to see whether the initiation of angiogenesis and revascularisation of the transplants occurred primarily in vessels of the transplant or in the underlying muscle bed. Initiation of angiogenesis and revascularisation were related to the initiation of myogenesis in the transplants, as indicated by the presence of autoradiographic labelling of nuclei (presumptive satellite cells) within degenerating muscle fibres.

Results of the present study show that vascular sprouting from transplants is obviously not an important mechanism in revascularisation of the mouse EDL. By contrast, the blood vessels of small muscle transplants in hamster cheek pouches send sprouts into the underlying skeletal muscle (Faulkner *et al.* 1983). The difference in these two studies may be accounted for by the different size of the transplants. Faulkner *et al.* (1983) used small fragments of EDL muscles containing approximately 20 fibres. The EDL transplants used in the present study were whole muscles containing approximately 1500 fibres. The single labelled endothelial nucleus observed at 36 hours in the present experiment may have survived in a similar way to those in the hamster transplants (i.e. due to nutrient diffusion) but only because of its position at the very edge of the transplant.

Contrastingly, during the period from 36 to 120 hours post-transplantation, activated endothelial cells were seen consistently, both within, and at the periphery of, the TA muscle over which the EDL muscles were transplanted. It was assumed that these activated cells were producing vascular sprouts passing towards the transplanted EDL muscles to establish connections with vessels in the transplants. However, in all

sections from the 52 transplants examined there was no direct evidence of any vascular connections between the underlying TA muscle and the EDL transplant. This lack of connections is probably due to the orientation of the blood vessels on the surface of the TA muscle, where the capillaries normally lie parallel to the surface, having few (if any) branches (Dawson, Tyler & Hudlická, 1987).

These results differ from those reported by Hansen-Smith *et al.* (1980) who found that transplanted EDL muscles in the rat were beginning to be revascularised peripherally on the second day (48 hours) after transplantation. This difference may be accounted for by the different sites of transplantation. Hansen-Smith *et al.* reimplanted the muscle into its original bed while in the present study the muscle was relocated to a new site over the intact TA muscle. The model used by Hansen-Smith *et al.* would have both damaged and stimulated the blood vessels exposed at the site of transplantation, making them immediately available to grow towards the transplant, and to anastomose with any surviving peripheral blood vessels. In our study, where the EDL muscle was transplanted to a new site, there was minimal tissue damage (compared with implantation in the same site) and very few, if any, blood vessels in the underlying muscles were exposed, the fascial coverings of the TA muscles remaining intact. That is, new blood vessels would have had to sprout and grow out from surrounding tissues. Furthermore, the intact epimysium surrounding the EDL transplants may have impeded the growth of capillaries from surrounding tissues into the transplant, further delaying the activation of endothelial cells within the transplant.

Was the lack of labelling in endothelial cells of the transplants until 96 hours due to unavailability of 3H-TdR to proliferating cells because of diffusion barriers between the underlying TA muscle and EDL transplant? In other words, endothelial cells in the transplant may have been proliferating but were unable to incorporate labelled thymidine. Such a diffusion barrier was not operative because 3H-TdR labelling occurred in cells (presumptive satellite cells) within degenerating muscle fibres in the transplant as early as 48 hours after transplantation (compare Fig. 1, endothelial labelling, with Fig. 4, muscle labelling). A possible explanation for this lack of labelling in endothelial cells of the transplant is that the endothelial cells were unable to survive the initial lack of nutrients following transplantation. Satellite cells (which are reserve myoblastic cells and can become activated when muscle fibres are damaged) on the other hand are obviously quite robust and can withstand severe trauma and prolonged ischaemia because they survive in muscles in which all fibres have been destroyed by mechanical damage, by myotoxic snake venoms or myotoxic anaesthetic agents such as Bupivacaine (Harris, Johnson & Karlsson, 1975; Carlson, 1976; Snow, 1977; Harris & Johnson, 1978). By comparison, there are no similar reserve endothelial cells and they may not withstand the ischaemic trauma of transplantation.

Hansen-Smith & Carlson (1979) reported that phagocytic cells (neutrophils and macrophages) move just ahead of blood vessels sprouting towards transplanted muscles. Although macrophages were first seen in transplants removed 72 hours after implantation in the present study, this was not considered to be conclusive evidence that revascularisation of the transplants was about to commence. These phagocytic cells may have simply migrated into the area from surrounding tissues as a result of the inflammatory response to tissue damage. Labelled cells adjacent to muscle fibres (presumptive satellite cells) were often seen from 48 hours through to 192 hours after transplantation, although initially only around the periphery of the transplant to a depth of approximately 150 μm, the zone which contained some surviving fibres. These results correlate with the activation time of presumptive myogenic cells in

similar EDL transplants, determined in a previous study (Roberts *et al.* 1989) where myogenic cells began to synthesise DNA and proliferate between 42 and 48 hours after transplantation. It was through the examination of the transplants, which had been allowed to regenerate for 14 days (in this previous study), that it could be determined that there was a ring of surviving muscle fibres around the periphery of the transplants, a phenomenon previously reported by Gulati, Reddi & Zalewski (1982) and Carlson & Faulkner (1983). The fact that these satellite cells began synthesising DNA and proliferating (at 42 to 48 hours) prior to a functioning vascular supply being established in the transplants (after 72 hours) would indicate that they were stimulated to proliferate by the diffusion of nutrients and mitogens from surrounding tissues, as discussed by Carlson & Faulkner (1983). Furthermore, there was no evidence of macrophages within the transplants at the time of satellite cell activation, indicating that contact between macrophages and satellite cells/myofibres is not essential for the initiation of myogenesis. However, this does not preclude the hypothesis that phagocytic cells may secrete some activating factor which diffuses into the transplants along with other nutrients, thereby initiating myogenesis (Moore, 1979). This may be further evidenced by the fact that the satellite cells were activated only in the region that contained surviving (as well as degenerating) myofibres, which were presumably within the distance limits of nutrient diffusion.

SUMMARY

The activation time of endothelial cells and myogenic cells (presumptive satellite cells) in small skeletal muscle transplants was examined, as well as in the muscle bed underlying the transplants, using autoradiography and light microscopy. At varying intervals, from 12 to 336 hours (14 days) after transplantation, the transplants and some of the underlying muscle were removed from two or four mice (at each interval), after the injection of each animal with tritiated thymidine 1 hour prior to transplant removal. Thus premitotic cells synthesising DNA were labelled.

Only one labelled endothelial cell nucleus was seen in all 20 transplants examined during the first 60 hours. Contrastingly, labelled endothelial nuclei were plentiful in muscles underlying the transplants from 36 hours post-transplantation. There was no evidence of a functional vascular supply in the transplants until 72 hours when one of the eight transplants examined at this time showed very slight peripheral revascularisation, with these peripheral vessels containing labelled endothelial cell nuclei. Ninety six hours after transplantation half the transplants sampled showed peripheral revascularisation, with these vessels containing labelled nuclei, and by 120 hours all transplants showed functional blood vessels and contained labelled endothelial cell nuclei. By 14 days after transplantation revascularisation and myogenesis were complete with only an occasional labelled nucleus seen. Autoradiographically labelled premitotic muscle nuclei (presumptive satellite cells) were observed in transplants from 48 hours after transplantation.

These results show that revascularisation of the transplanted muscles is not necessary for the activation of myogenic cells, but that activation is probably due to some other stimulus, possibly the diffusion of nutrients from blood vessels of the adjacent tissues.

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