The Influence of Revascularisation, Vasoactive Drugs and Exercise on the Regeneration of Skeletal Muscle, with Particular Reference to Muscle Transplantation.

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Abstract

This review, concentrating on the aspects mentioned in the title, discusses the process of revascularisation (and the influence of vasoactive drugs and exercise on this process) and regeneration of mature skeletal muscle, with particular reference to muscle transplants. The effects of physical treatments such as exercise and of vasoactive drugs on the vascularity of both normal and regenerating skeletal muscle are evaluated. Unpublished data are also presented.

Key words: Skeletal muscle, satellite cells, revascularisation, regeneration, transplantation.

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In all major reviews of skeletal muscle regeneration [3, 13, 15, 67] it is agreed that no matter what the cause of injury, the essential processes involved in regeneration are similar; however, the timing and outcome are variable due to the types, extent and severity of the injury. In this review emphasis will be placed on transplantation of "free" whole intact muscles as a model of muscle precursor cell activation, revascularisation and regeneration, although findings from other models will be discussed in relation to these processes.

The terms used here to describe the myogenic cells responsible for muscle regeneration are similar to those used by Robertson *et al.* [61], unless otherwise stated. The general term "mononuclear muscle precursor cells" (mpc) will be used to encompass satellite cells, presumptive myoblasts and myoblasts.

Although it has been postulated that there might be more than one possible source of the mpc responsible for regeneration [discussed in: Grounds, 32, 33], the satellite cell of skeletal muscle, as described by Mauro [53] will only be considered here, as descriptions of other possible sources are beyond the scope of this review. The satellite cell is essentially defined on the basis of its position between the plasmalemma (sarcolemma) and external (basal) lamina of muscle fibres.

Thompson [74] described the successful clinical use of free intact muscle transplants; subsequently whole muscle transplants (either free or with neurovascular anastomoses) have been used clinically to treat disorders such as anal and urinary incontinence [24, 34], palsies of the facial muscles [38, 39] and the repair of injury to muscles of the upper limb [25, 51, 66]. The free muscle transplant is also used extensively as an experimental animal model to study the processes of muscle regeneration [for reviews see: 3, 13, 15, 67].

Following transplantation, degeneration of the myofibres commences, and this process continues even when the next phase of the regeneration process, the infiltration of neutrophils and macrophages into the muscle transplant, commences. Until recently, it was believed that at this stage, satellite cells became activated and synthesized DNA in preparation for cell division. However, a recent autoradiographic study by Roberts and McGeachie [58] has shown that satellite cells in whole muscle transplants become activated prior to the reintroduction of a vascular supply, and in the absence of phagocytic cell infiltration. This suggests that there might be a diffusion of nutrients and mitogens (which stimulate mpc) into the transplant from intact muscle or the vasculature surrounding the transplant, or from inflammatory cells accumulating in adjacent tissues. Alternatively, an intrinsic factor may be produced within the damaged muscle. There is evidence that an intrinsic factor is present in both uninjured and injured muscle. This factor, known as Bischoff's Growth Factor, acts as both a mitogen for satellite cells [7, 8, 9, 10] and as an angiogenic agent [56]. This factor appears to

have similar properties to basic Fibroblast Growth Factor [10]. The possible *in vivo* roles of this growth factor and others like Platelet Derived Growth Factor (PDGF) and the Insulin-like Growth Factors in muscle regeneration have been reviewed recently by Grounds [33].

Revascularisation

If a transplanted muscle does not become effectively revascularised, it will not regenerate. This is evident in muscle transplants (without any neurovascular anastomoses) weighing > 3g, where the central core of the transplant eventually becomes fibrotic [14, 15, 45, 52, 76]. This is presumed to be because the satellite cells in this central region cannot survive the prolonged ischaemia and reduced oxygen tension in the transplant core, conditions which favour the proliferation of fibroblasts [73]. Schultz, Albright, Jaryszak and David [70] examined transplants at various times (0-96 hours) after transplantation and found no evidence of satellite cell death in this ischaemic core region. Their observations, in conjunction with evidence from other researchers, suggest that satellite cells may migrate away from this central region to oxygen-rich areas of the transplant and may subsequently migrate back to the core as revascularisation proceeds [6, 55, 68, 69, 70].

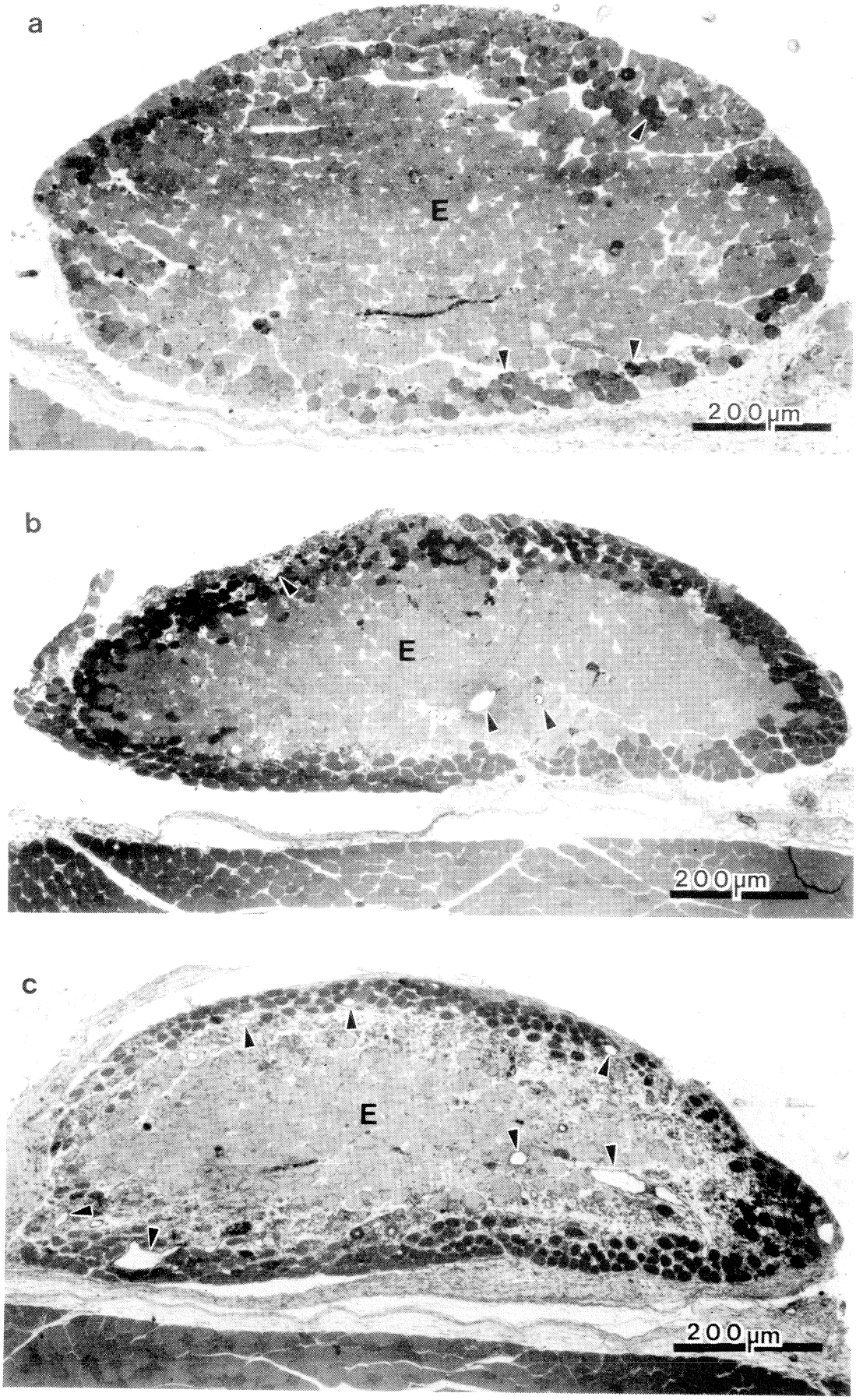
The ischaemia which causes degeneration of muscle fibres in a transplant, is paralleled by degeneration of the vascular system; with degeneration of the endothelial cells of the capillary bed, and fragmentation of endothelial cells and some smooth muscle of the larger vessels [35, 36]. There appeared to be no degeneration of the basal laminae of blood vessels, when examined at the electron microscopic level [36].

The process of revascularisation commences when phagocytic cells (neutrophils and macrophages) enter the muscle transplant, and begin to ingest necrotic cell debris. These phagocytic cells precede endothelial tubes, which have sprouted from surrounding tissues and enter the periphery of the transplant [36]. In the rat revascularisation of the transplant begins late on the second day following transplantation [36] and late on the third day in the mouse [58; See Fig. 1). Capillaries sprout from these tubes approximately one day after entering the transplant, before differentiating into arterioles and venules [36]. As the periphery of the transplant becomes revascularised, sprouting endothelial tubes utilise the patent basal laminae of existing damaged blood vessels to shunt blood towards the centre of the transplant. In the rat, a vascular system is seen throughout the transplant by 6 to 8 days [36] and in the mouse by 8 days [58].

This is not the only means by which revascularisation can occur. It was shown by Faulkner, Weiss and McGeachie [23] that in very small muscle grafts in the hamster cheek pouch, surviving blood vessels sprouted from the graft; these vessels anastomosed with vessels growing out from surrounding tissues. However, these were very small grafts (approximately 20 fibres) and it is probable that all blood vessels in the graft were able to survive the transplantation ischaemia, because of nutrient diffusion from the surrounding tissues, and were thereby capable of sprouting. In larger transplants almost all the blood vessels die due to the ischaemia associated with transplantation. Roberts and McGeachie [58] reported few viable peripheral endothelial cells in mouse EDL transplants examined autoradiographically (prior to transplant revascularisation).

This leads to the question "What initiates the growth of blood vessels into the avascular transplants?" The stimulus for the revascularisation process to begin is unknown, but it is likely that there are a number of factors which interact. Phillips and Knighton [56] have reported that growth factor extracted from injured skeletal muscle [7, 8, 10], has angiogenic properties and acts in a dose dependent manner. Further, macrophages can secrete angiogenic factors when cultured under low oxygen tension, a situation which mimics the ischaemic hypoxia following injury [26, 42]. One of the most potent of these angiogenic agents is

Figure 1. a-f, are all low power photomicrographs (TS) of mouse EDL muscle transplants (E), placed over the TA muscle, at various times after the transplants were inserted. 1a. An EDL transplant removed 48 hours (2 days) after insertion. No evidence of revascularisation can be seen, but there is a peripheral rim of surviving muscle fibres, surrounding an ischaemic core. Interspersed among the surviving fibres are fibres containing large dark staining lipid droplets (arrows); these are fibres in a "marginal" metabolic state, as described by Carlson and Faulkner [15]. Satellite cells were synthesizing DNA in this transplant [58]. 1b. An EDL transplant removed 72 hours (3 days) after transplantation, showing the first signs of revascularisation (arrows). The peripheral rim of surviving fibres is now more clearly delineated. 1c. A transplant removed 96 hours (4 days) after insertion, showing that revascularisation is continuing (arrows), with large sinusoidal blood vessels being visible. At this time, small immature myotubes are seen for the first time. 1d. By 144 hours (6 days), there is a much reduced central core to the EDL transplant (E), and three zones are clearly distinguishable from the periphery inwards: a surviving rim of muscle fibres, a zone containing myotubes at varying stages of maturity and an inner zone which has still not been fully revascularised. 1e. At 192 hours (8 days) the EDL transplant (E) is fully revascularised, with a concentric gradient consisting of outer surviving myofibres, with progressively less mature myotubes visible as the centre of the transplant is approached. At this time the transplant is completely revascularised. 1f. Fourteen days after transplantation the EDL (E) has regenerated, and consists of surviving peripheral fibres which are reduced in size due to denervation atrophy (compare with TA muscle fibres (T)). The majority of the transplant area consists of myotubes of varying diameters and maturity. Notice the persistence of abnormally large blood vessels in the transplant (arrows).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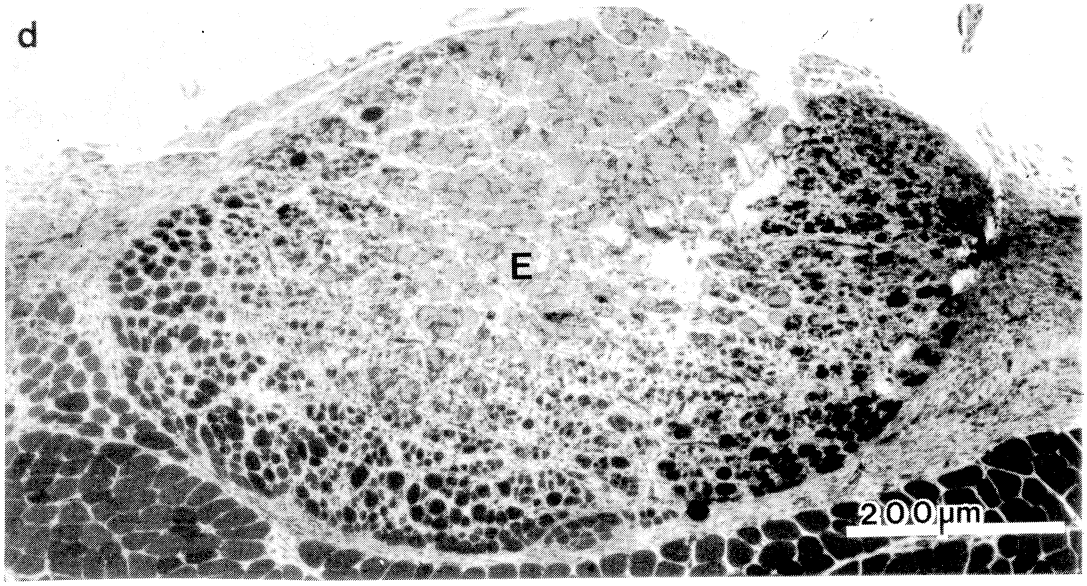
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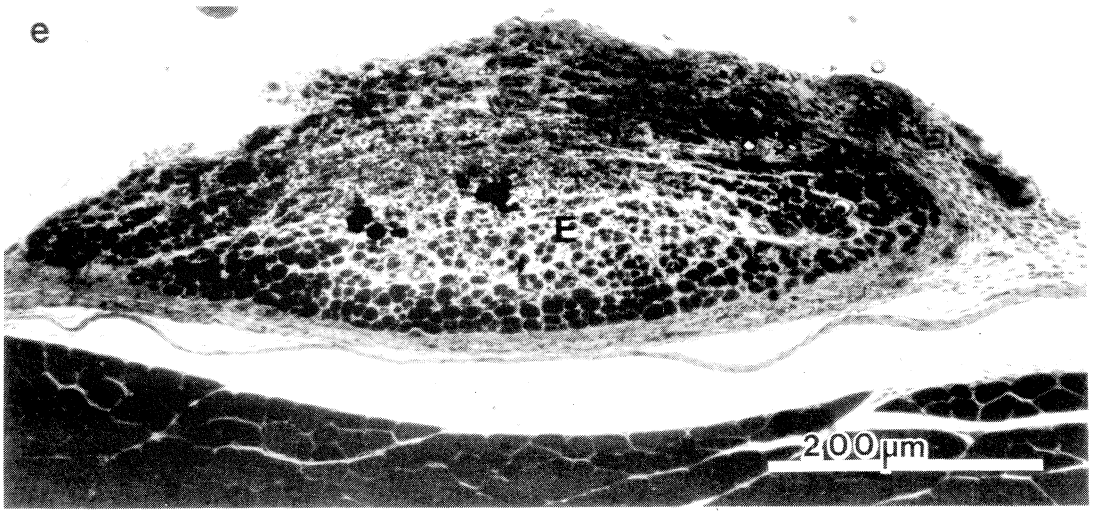
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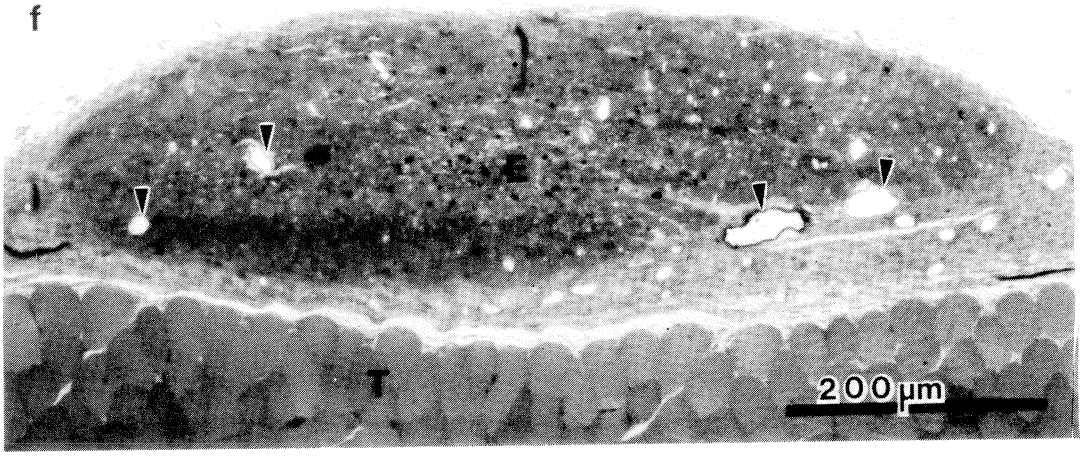
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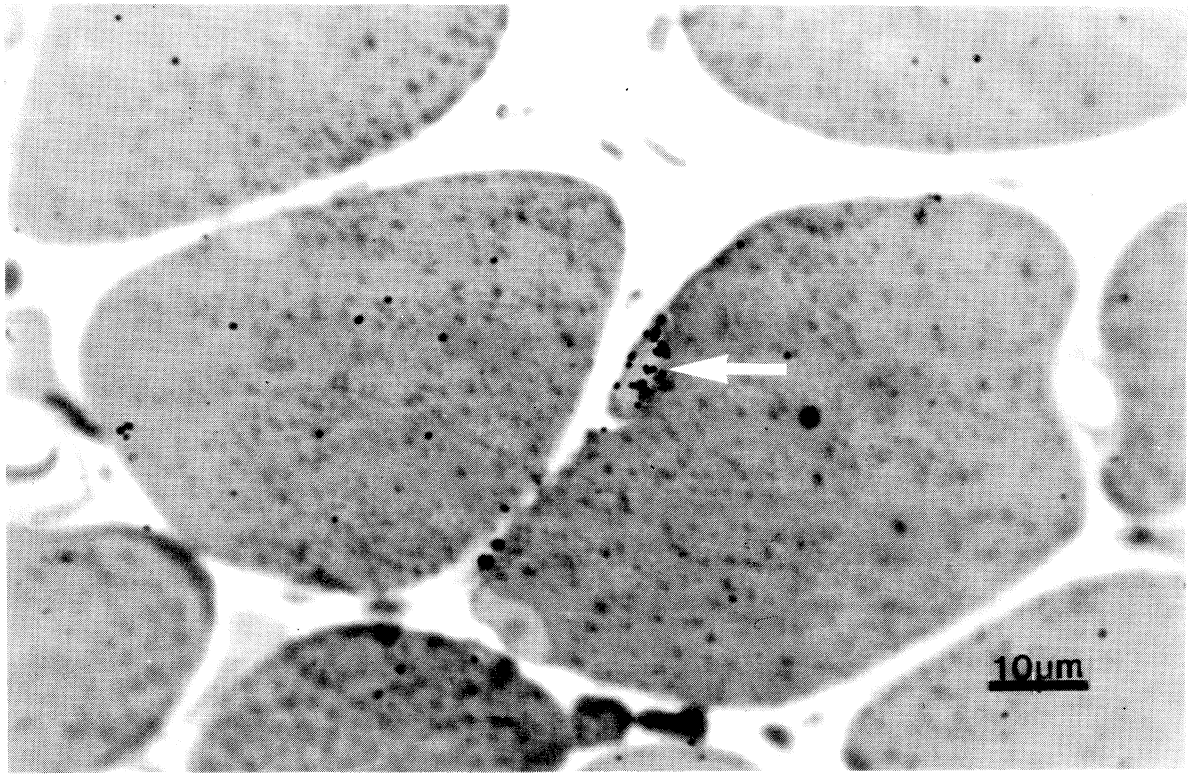

Figure 2. An autoradiographically labelled juxtasarcolemmal cell, presumably a satellite cell (arrow) in a muscle fibre at the periphery of a transplant removed 36 hours after insertion. The animal from which the transplant was removed received twice daily injections of clenbuterol (300µg/kg) following transplantation until sacrifice, and received a single injection of tritiated thymidine one hour prior to sacrifice.

probably FGF [30]. Additional FGF is bound to the extracellular matrix of muscle fibres by heparan sulphate proteoglycans [80] which can be released by heparinases, produced by (among other cells) macrophages [41].

As Burton, Carlson and Faulkner [12] state *"The regeneration of fibres within a muscle graft relies ultimately on the degree to which the graft is revascularised."* This leads to the conclusion that if revascularisation could be accelerated or enhanced, fibre regeneration may also be improved. As Grounds [33] states *"Clearly, factors which attract macrophages to an injury or, in larger injuries stimulate revascularisation, will enhance muscle regeneration."*

Factors which influence revascularisation

In normal muscle an increase in capillary formation (neovascularisation) can be induced in a number of ways, such as exercise [1, 4, 11, 16, 46], chronic long-term electrical stimulation [18, 65], cold temperature [71] and the long-term administration of β_2-agonists [72]. The influence of exercise and the β_2 agonists will be discussed in the following section.

Exercise

As well as the neovascularisation mentioned above, another response to prolonged exercise is hypertrophy (increase in size) of the muscle fibres [4, 16]. Debate continues over the basis of this response: is it an increase in the size of the muscle fibres (hypertrophy), or hyperplasia (an increase in numbers of fibres and myonuclei) or a combination of both? Increased numbers of muscle fibres (hyperplasia) have been reported in weight-trained cats [28], but initial reports of adaptive growth in muscles stimulated by ablation of synergists showed no change in fibre numbers [27]. Other reports have indicated that both hypertrophy and hyperplasia occur [29, 63, 80]. Schmalbruch [67] asserts that the response is not due to hyperplasia, but an increase in the size of individual fibres, with any new fibres being the result of regeneration of injured fibres damaged during exercise. In the light of information presented later in this review, recent experimental evidence supports Schmalbruch's view. However, White and Esser [79] conclude that *"for different models of activity-induced skeletal muscle growth, there is evidence of muscle fibre hyperplasia and/or hypertrophy. Clearly,*

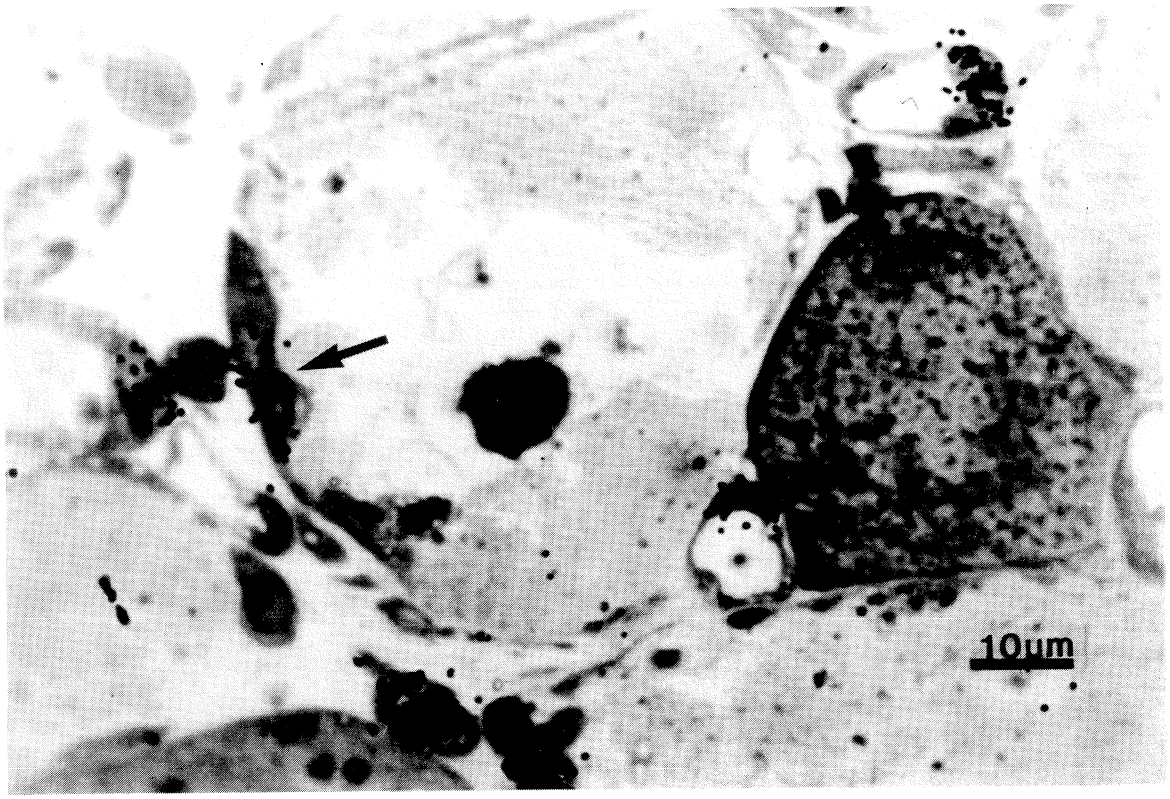

Figure 3. The periphery of an EDL muscle transplant removed 72 hours after insertion. The mouse from which it was removed had received twice daily injections of clenbuterol (300µg/kg) from the time of transplant insertion until sacrifice, and was given a single injection of tritiated thymidine one hour prior to sacrifice. Note the remnants of a degenerating muscle fibre with a macrophage ingesting necrotic debris. Also within the fibre is an autoradiographically labelled probable satellite cell nucleus (arrow), and three perfusion-cleared capillaries, the endothelial nuclei of two being autoradiographically labelled.

skeletal muscle fibres possess the appropriate mechanisms for both responses".

A single exercise session can cause damage to skeletal muscle fibres [21, 43, 64]. This finding supports those of Irintchev and Wernig [40] who found that in different strains of mice undergoing voluntary running, it was at the beginning of the exercise regime that injury occurred. It has also been shown that exercise can induce an enhanced phagocytic response [21, 54] and the activation and proliferation of satellite cells [21, 54].

Satellite cell activation may be due to a number of factors following this exercise-induced injury. Darr and Schultz [21] postulate that it may be the release of mitogenic factors from the basal lamina of the injured fibres, such as Bischoff's Growth Factor [7, 8, 9, 10] or FGF [80]. That exercise can increase levels of a mitogenic factor in both serum and individual muscles has been shown by Chromiak *et al.* [17]. These researchers provide evidence showing that this factor is probably not FGF, as the myoblastic cell line on which this factor was tested is unresponsive to FGF. It may also be that if the exercise is sustained and blood flow to skeletal muscles increased, that blood-borne mitogens and nutrients may activate the satellite cells. However, it is probable that a combination of these factors induces this mitotic activity.

Experiments into the effects of exercise on skeletal muscle grafts with implanted nerves in rats has shown that endurance exercise, initiated 28 days after the grafting procedure (at a stage when regeneration would be complete and the new muscle fibres are growing), can induce an increase in muscle mass and protein content of the graft [77, 78]. Although there is an increase in fibre cross-sectional area (for Type I fibres) in these grafts with time, this difference declines until there is no difference between normal control muscle and the trained and untrained grafts [19]. When rats with similar nerve-implanted grafts were run- trained (from 28 days post-grafting) before ablation of synergistic muscles, growth of these grafts was impaired [22].

Experiments designed to investigate the effects of exercise on the revascularisation and regeneration of muscle transplants were carried out by Roberts and McGeachie [59]. These experiments increased blood flow to, and exercised the muscles underlying muscle transplants, to

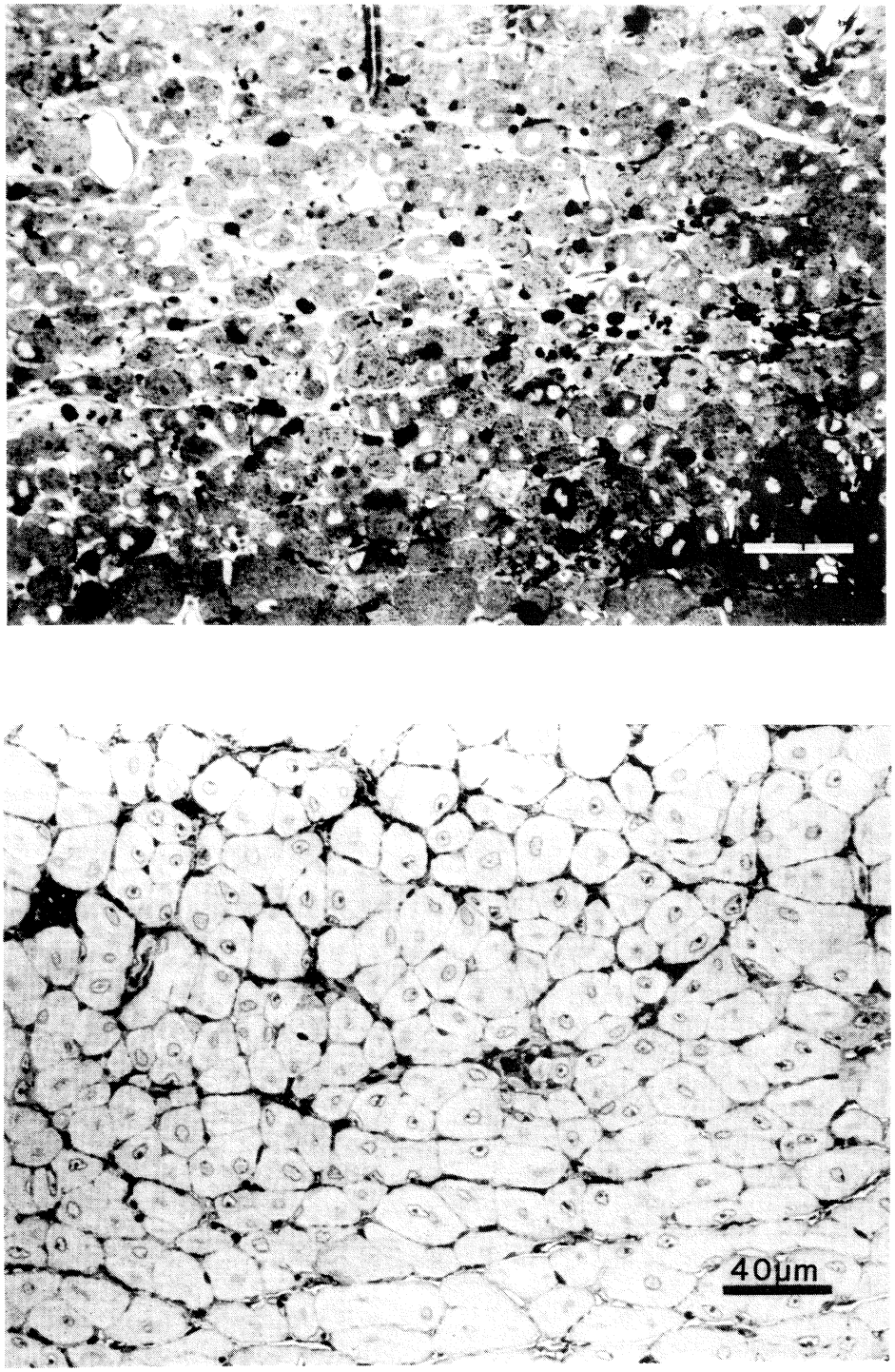

Figure 4. Two photomicrographs taken from the central areas (TS) of an untreated EDL transplant (upper) and a clenbuterol treated (lower) EDL transplant (300µg/kg) which had regenerated for 14 days. The upper photograph, where the tissue was stained with p-phenylenediamine shows many small myotubes with characteristic centrally located nuclei, the myotubes being separated by large areas of interstitial space, interspersed with some relatively large blood vessels. The lower photograph (the section was stained with methylene blue) once again shows myotubes with centrally located nuclei. However the area occupied by interstitial space is negligible when compared with the upper photograph. This is due to the larger volume of the transplant being occupied by muscle cytoplasm.

determine if it was possible to increase nutrient diffusion to the initially avascular transplants, enhance revascularisation of the transplants, and determine the effects on transplant regeneration. Mice ran voluntarily (daily) for two weeks prior to receiving bilateral free transplants of the extensor digitorum longus muscles. Mice returned to voluntary exercise within hours after transplantation and continued running until sacrifice. It was found that satellite cells in the periphery of the transplants were synthesizing DNA 30 hours after transplant insertion. This was 18 hours earlier than in non-exercised transplants, where satellite cells began DNA synthesis 48 hours after the surgical procedure [57, 58]. In addition, the exercised transplants showed evidence of revascularisation beginning 60 hours after insertion, as opposed to 72 hours in non-exercised animals [58]. When regenerated transplants from both exercised and non-exercised transplants were compared morphometrically, a significant increase was found in both the overall size of the exercised transplants, and their vascularity. In addition to the effects of exercise on revascularisation and regeneration, the effects of various vasoactive drugs were also investigated.

Catecholamines.

This section refers not so much to the naturally occurring catecholamines: adrenaline, noradrenaline and dopamine [47], but to synthetic analogues of these substances which mimic their effects and are known as the sympathomimetics. The catecholamines' role is in the control of the autonomic nervous system, acting on the α and β adrenoceptors of the adrenergic system [2, 44]. The sympathomimetics affect these same α and β receptors, having either agonistic or antagonistic (opposite) effects. It is the influence of drugs acting on the β_2 receptors (β_2 agonists) which have stimulated the interest of muscle researchers, due to the preponderance of these receptors on skeletal muscle, as well as their effect on smooth muscle of the vascular system. The main effect of the β_2 agonists is to relax the smooth muscle of blood vessels, thereby increasing blood flow, particularly in skeletal muscles; another effect on skeletal muscle is to cause tremor [47].

Although there are many drugs which act on the β_2 receptors, much research has been carried out using the β_2 agonists isoproterenol (isoprenaline) and clenbuterol. Chronic administration of isoproterenol has been shown to induce the formation of new blood vessels in normal skeletal muscle of rats [72] and it has been postulated that this is a result of the increased blood flow (agonist effect). However, when clenbuterol was administered chronically, the increased blood flow was transient [62].

Until recently there was no evidence of catecholamines affecting satellite cell activation, but it had been observed that adrenaline (at high concentrations) accelerated the fusion of primary chick myoblasts *in vitro* [20]. It has now been shown that isoproterenol and ractopamine (another, β_2 agonist) significantly enhance the proliferation of satellite cells removed from chick breast muscle [31]. These authors also found that when propranolol (a β antagonist) was added to the culture medium, the proliferation of satellite cells was significantly reduced. Grant *et al.* [31] interpreted this result as showing that it is the β adrenergic receptor which mediated the proliferation of satellite cells.

Clenbuterol. Compounds such as the β_2 agonist clenbuterol have been shown (in experimental animal models) to be capable of reversing or halting atrophy associated with muscle denervation [48, 49, 81], this reversal being associated with an increase in both DNA and protein content. Normal muscle treated with clenbuterol shows an increase in protein and RNA, but not DNA [50] and these authors suggest the DNA increase in denervated muscles is due to satellite cell activation, with the implication that clenbuterol stimulates their activation. Evidence has recently been presented showing that the effect of clenbuterol is to decrease the breakdown of myofibrillar protein [5].

In vivo, autoradiographic studies of whole muscle transplants in mice, treated daily with intraperitoneal injections of clenbuterol, after grafting, have shown that satellite cells can become activated in the periphery of transplants as early as 30 hours after transplant insertion (Fig. 2), with evidence of transplant revascularisation at 60 hours (Fig. 3), 12 hours earlier than in controls [60]. Whether this is a direct effect of the drug acting on satellite cells is not confirmed, but this is suggested by the data obtained. Alternatively, it might also be the direct effect of greater blood flow to the muscles underlying the transplant, resulting in a greater diffusion of nutrients and mitogens to the avascular transplant. Drugs of this type cause tremor in normal skeletal muscle [47], and it may be that prolonged treatment causes either a leakage of mitogens from muscles surrounding the transplants, due to overwork of the muscles [as hypothesized by Bischoff, 10]. Alternatively, it may be the release of factors within the transplanted muscle due to the mechanical disruption caused by the constant tremor in the surrounding muscles. Morphometric analysis of regenerated transplants and controls sampled 14 days after grafting showed a highly significant increase in the size of the muscle fibres of the clenbuterol treated transplants (Fig. 4). This may be attributed to either earlier satellite cell activation (and concomitant effects on the timing of new muscle fibre formation) or the known effects of the drug on muscle protein synthesis/degradation.

Isoproterenol. Experiments were also carried out to compare the effect of the β_2 agonist isoproterenol with those of clenbuterol. We were particularly interested to determine if chronic administration of isoproterenol could induce an earlier revascularisation of skeletal muscle transplants, (Roberts and McGeachie, Chronic application of isoproterenol enhances the revascularisation and regeneration of skeletal muscle transplants, manuscript in preparation). Isoproterenol was applied to a muscle transplantation model using the delivery method (daily injection of the drug in oil) of Sillau *et al.* [72]. Two experimental regimes were used: in one group ("pre- and post-transplantation" group), two weeks prior to transplantation, mice were given daily injections continuing daily through transplan-

tation until sacrifice. The second group ("post-transplantation group") received daily injections of isoproterenol from the day of transplantation until sacrifice. Evidence of revascularisation of the "post-transplantation" group was seen 48 hours after insertion, some 24 hours earlier than in controls. However, in the group which received the drug both prior to and following transplantation, evidence of revascularisation was not seen until 72 hours after transplant insertion, the same time as in controls. This indicates that the effect of isoproterenol on the vascular system is transient, as already discussed in relation to the chronic application of clenbuterol, and is probably related to the phenomenon of desensitization (a reduced response with each successive use of the drug, see Harden [37] for a review). When treated and non-treated transplants were analysed morphometrically following regeneration, there was a significant increase in the size of the drug-treated transplants (when compared with controls and non-treated transplants) in both groups. This significant difference was also observed in the treated groups, with a dose dependency being observed. The two groups which had received the drug prior to and following grafting were significantly larger than grafts from animals which had received isoproterenol only after the transplantation procedure. It must be reiterated that the before/after treatment resulted in no increase in the speed of graft revascularisation, but that grafts from animals which received isoproterenol only after surgery were revascularising earlier. This suggests that although the speed of revascularisation does not appear to be a limiting factor in this model, markedly increasing the speed of revascularisation in larger grafts, where revascularisation is known to be critical, may be of great clinical interest. Also, the effect on regeneration may not be linked solely to an earlier revascularisation, but also to a direct action of the drug on the muscle fibres or satellite cells, as seen with clenbuterol. It is possible that both of these situations may occur simultaneously.

Further observations on the influence of angiogenic agents on revascularisation result from studies using erucamide, a potent angiogenic factor composed of a 22 chain fatty acid, purified from bovine omentum [75]. Erucamide was incorporated into controlled-release polymers of Elvax and implanted into crush injured skeletal muscles of mice, for a 10 day period from the time of injury. Although an increase in blood vessel numbers was noted, there was no difference in the histology of muscle regeneration and the proportions of new muscle fibres and fibrotic tissue, when compared with controls which had been injured and sampled at the same time, but which had not been treated with erucamide (Mitchell, McGeachie, Grounds, Crawford and Chirila, 1991. Induction of angiogenesis by the lipid erucamide does not improve skeletal muscle regeneration. Submitted for publication).

Conclusions

The conclusions to be drawn from the material presented in this review are that when revascularisation is enhanced by physical or chemical means it does result in an earlier onset of DNA synthesis by satellite cells and increased size of the transplant: however this appears to be due to the regimes used to enhance revascularisation, rather than being a primary response to revascularisation itself. For example, exercise produces muscle damage and may release activating factors (either locally or systemically), vasoactive drugs may act on the β_2 receptors of muscle cells directly to stimulate cell replication, and these same drugs influence protein synthesis/degradation and can result in hypertrophy. However, it must be re-emphasized that without effective revascularisation, regeneration will be impaired. Most of the data presented in this review are obtained from studies of relatively small transplants in rodents. Application of the regimes used to enhance revascularisation may have significant clinical interest with respect to the larger muscles used in human transplantation procedures. Although revascularisation is of clinical interest, it is not the only limiting factor for effective muscle regeneration. Other factors appear equally important in the formation of new muscle fibres, such as those which activate muscle precursor cells, or can enhance muscle protein accretion.

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