Towards Understanding Skeletal Muscle Regeneration

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SUMMARY

Factors which effect proliferation and fusion of muscle precursor cells have been studied extensively in tissue culture, although little is known about these events in vivo. This review assesses the tissue culture derived data with a view to understanding factors which may control the regeneration of mature skeletal muscle in vivo. The following topics are discussed in the light of recent developments in cell and molecular biology:

1) Injury and necrosis of mature skeletal muscle fibres
2) Phagocytosis of myofibre debris
3) Revascularisation of injured muscle
4) Activation and proliferation of muscle precursor cells (mpc) in vivo Identification of mpcs; Satellite cell relationships; Extracellular matrix; Growth factors; Hormones; Replication.
5) Differentiation and fusion of muscle precursor cells in vivo Differentiation; Fusion; Extracellular matrix; Cell surface molecules: Growth factors and prostaglandins
6) Myotubes and innervation.

Introduction

The ability of mature skeletal muscle to regenerate has been documented since the second half of the 18th century. Investigations made late in the 19th century showed that in a range of species muscle did have great powers of regeneration in response to chemical and physical injury. The most important of these early observations were those of Zenker (1864), Waldeyer (1865), Weber (1867), Neumann (1868) and Volkmann (1893), whose findings have been well reviewed and have been confirmed by much subsequent work in recent years. There have been many excellent descriptions of skeletal muscle regeneration after injury or transplantation (reviews), although little is known about the cellular interactions which control this process. The extent and success of regeneration varies with the nature of the injury, but in all situations the process involves revascularisation, cellular infiltration, phagocytosis of necrotic damaged muscle, proliferation of muscle precursor cells (mpc) and their fusion (either into multinucleated myotubes or with the ends of damaged muscle fibres) and finally, re-innervation.

Recent developments in cell and molecular biology, in particular the role of various growth factors, have begun to provide some insight into the subtle interaction and complex events which control these well documented events. Most of these studies have been carried out in tissue culture using developing muscle or muscle cell lines. The relevance of these observations to understanding the process of regeneration of mature skeletal muscle in vivo is examined in this review.

1. Injury and Necrosis of Mature Skeletal Muscle Fibres

It is well documented that, after many forms of injury ranging from focal damage to transplantation of whole intact muscles, the basal lamina of muscle fibres remains intact. The integrity of the basal lamina is important for successful regeneration as it serves as a scaffold for new myotube formation and its presence minimises fibrosis; however, muscle regeneration does occur after severe physical injury such as crush lesions, or transplantation of minced muscle fragments where the basal lamina is
severely disrupted. It is the extent of damage to the plasmalemma that provokes the necrosis of the muscle fibre, and the necrosis probably results mainly from elevated intracellular calcium levels and also to activation of complement. That necrosis results from disturbances to intracellular calcium levels is well established, as excess calcium inhibits the normal respiratory activities of mitochondria, activates calcium-dependent neutral protease, depolymerises microtubules, and overwhelms the calcium uptake capability of the sarcoplasmic reticulum. The activation of complement and production of C5b-9 membrane attack complex has been demonstrated on membranes of damaged muscle fibres and results in muscle cell lysis; other components of complement activation serve as powerful chemotactic and stimulating agents to macrophages, leading to the efficient removal of necrotic tissue.

To minimise necrosis after injury, the damaged portion of the muscle fibre must be rapidly sealed off from the nearby undamaged sarcoplasm. The process by which demarcating membranes are formed after focal injury has recently been investigated by two groups. Electron microscopic studies after superficial injury by Karnovsky’s fixative to tibialis anterior muscles of mice, show the accumulation of membrane vesicles in the structurally intact part of the myofibre, and the presence of many interdigitating membranes at the interface with the damaged portion by 9 h: apparently continuous new plasmalemma to seal off the injured area was formed by 12 h after injury. Our general observations are similar to those of Carpenter and Karpita in a related electron microscopic study after focal injury by needle puncture to gastrocnemii muscles of rats, except that they report demarcating membranes from 3 to 7 h after injury. We consider that these early membranes represent fragments of collapsed plasmalemma and are not incomplete and temporary structures, and that new demarcating membranes sealing the injury are assembled de novo and are not completed until 9 to 21 h after injury.

2. Phagocytosis of Myofibre Debris

Phagocytosis of damaged muscle is a very important event preceding effective muscle regeneration, regeneration being inhibited by persisting necrotic tissue. The removal of the necrotic muscle is largely the result of phagocytosis by macrophages. Polymorphonuclear leucocytes, which are also phagocytic, are seen beneath the basal lamina inside damaged murine fibres within 3 h after focal injury, and macrophages are present by 6 h: the proportion of macrophages increases after this time and by 24 h polymorphonuclear leucocytes are no longer conspicuous. After extensive injury or muscle transplantation the presence of such cells is limited by, and slightly precedes, revascularisation of the damaged tissue. Clearly, factors which attract macrophages to an injury or, in larger injuries stimulate revascularisation, will enhance muscle regeneration.

After focal muscle injury in mice, there was an accumulation of mitochondria and other cell organelles by 6 h immediately adjacent to, but lying outside the zone of new membrane formation. The mitochondria were excluded from the sealed undamaged sarcoplasm, and lay in the necrotic zone where they were phagocytosed by macrophages. Since mitochondrial membranes from human heart muscle activate complement in vitro, it is possible that the accumulation of mitochondria at the interface of the necrotic and healthy myofibres activates complement and thereby attracts macrophages enhancing their function in these areas. It has long been recognised that macrophages play a central role in muscle regeneration as they are not only responsible for phagocytosing the necrotic muscle, but they also secrete a wide range of products which directly or indirectly modulate the behaviour of muscle precursor cells (see Section 4).

The accumulation of macrophages at sites of tissue damage is universally important after tissue damage. Complement cleavage products are produced after cell injury by a range of stimulants, and C5a is well recognised as an important chemoattractant for leukocytes. Other chemoattractant mediators develop from metabolites of arachidonic acid (e.g. leukotriene B4) released from the breakdown of membrane phospholipids, and cell-derived protein factors produced by lymphocytes, polymorphonuclear leukocytes, mast cells and platelets. Potent chemoattractive activity for monocytes has been described for human α-thrombin, and other chemoattractive factors may be derived from kinin-forming, fibrin-forming, and fibrinolytic pathways in blood vessels damaged at the site of injury. Changes in levels of Ca2+ and proteases are often involved in the production of chemoattractants, and lower doses of chemoattractants are chemotactic, compared with the dosage required to activate and stimulate the secretory responses of phagocytic leukocytes.

3. Revascularisation of Injured Muscle

As in general tissue damage, revascularisation is an important event for ensuring successful new muscle formation after severe injury or in transplanted muscles. In transplants of whole intact muscle in which neurovascular connections are completely disrupted, it is known that some muscle fibres at the periphery do not degenerate, and their survival is presumably due to diffusion of nutrients and gases from vessels in adjacent tissues. New muscle formation in such grafts is normally excellent suggesting that muscle precursors are very resistant to ischemia. It has been demonstrated that during regeneration the central portion of whole muscle grafts becomes devoid of viable muscle precursor cells (satellite cells), but it is not known whether these cells die and are replaced by other muscle precursors migrating inwards from peripheral regenerated zones, or, alternatively, whether the precursor cells temporarily migrate out from the ischemic central zone to the periphery, and subsequently return as the central areas become revascularised.

In very large grafts the central zone does not regenerate successfully and becomes fibrous. This may be due to prolonged ischemia and low oxygen tension favouring the
proliferation of fibroblasts\textsuperscript{197}. It seems likely that the efficiency of revascularisation generally relates to the extent of fibrosis after muscle injury. A range of factors are known to stimulate revascularisation usually by increasing endothelial cell proliferation. Macrophages grown under conditions of low oxygen tension, similar to hypoxic conditions after injury, secrete angiogenic factors\textsuperscript{61,107}. One of these angiogenic factors is basic fibroblast growth factor (FGF)\textsuperscript{64,93,103}, which powerfully stimulates neovascularisation \textit{in vitro} in a range of situations\textsuperscript{100}. Additional FGF is present within muscle bound to heparan sulphate proteoglycans\textsuperscript{10,14,101,220} and glycosaminoglycans\textsuperscript{167} of extracellular matrices and can be released by the action of heparinases secreted by lymphocytes and macrophages\textsuperscript{102} and by plasmin\textsuperscript{167}.

FGF is also produced by endothelial cells in a self-stimulating autocrine manner\textsuperscript{34,103}, and their response to FGF is modulated by the extracellular matrix\textsuperscript{93}. Apart from FGF, a range of other growth factors are known to stimulate revascularisation, including the chemottractants referred to in the previous section and prostaglandin E\textsubscript{2}\textsuperscript{50}. In addition, a unique factor which is a specific mitogen for skeletal muscle precursor cells, and is extracted from uninjured and injured muscle\textsuperscript{12,14} is also highly angiogenic\textsuperscript{136}.

Conditions which increase capillary growth within normal undamaged muscle have similar implications to an enhanced angiogenic response, as the increased density of blood vessels and blood flow will enhance the availability of leukocytes if such tissue is damaged. Capillary growth is increased in skeletal muscle subjected to repeated endurance training, chronic low frequency electrical stimulation\textsuperscript{21} or cold\textsuperscript{199}. In addition, capillary growth can be stimulated in muscle by drugs like isoprenaline\textsuperscript{590}. The proposal that conditions which enhance capillary growth might accelerate revascularisation and the presence of macrophages is supported by experiments in mice, where isoprenaline was injected daily after allotransplantation of whole intact extensor digitorum longus (EDL) muscles (Roberts and McGeezie, manuscript in preparation). Infiltrating macrophages and revascularization were conspicuous in regenerating grafts at 48 h, whereas these events in similar (control) grafts made into untreated mice are not normally seen before 3 days\textsuperscript{168,169}.

4. Activation and Proliferation of Muscle Precursor Cells (mpc) \textit{in vivo}

Identification of mpc

The assumption is widely made that precursor cells of mature muscle are derived exclusively from satellite cells\textsuperscript{23,185}; however, satellite cells are defined essentially on their geographical location between the plasmalemma and external lamina (widely referred to as the basal lamina) of muscle fibres (Fig. 1A) and they may be "impersonated" by other cell types\textsuperscript{245}. To date there appears to be no marker that can specifically identify quiescent (or recently activated) mpc \textit{in vivo} (Fig. 1B) and it may be that specific antigens are not produced by such cells. The monoclonal antibody 5.1 H 11 which is specific for activated human (but not rodent satellite cells) does not bind to quiescent satellite cells of normal or denervated human muscle\textsuperscript{91}; it has recently been demonstrated that this antibody recognizes the cell surface adhesion molecule N-CAM\textsuperscript{211,18}. Other promising monoclonal antibodies have been described as being specific for satellite cells extracted from adult chicken muscle\textsuperscript{217}. The SAT 2 HIO antibody reacts with mononucleated cells closely associated with the basement membrane of myofibres \textit{in vivo}: these SAT 2 HIO positive cells may be satellite cells but this requires confirmation at the EM level (Yablonka-Reuveni, personal communication). In addition, SAT 2 H 10 reacts with smooth muscle cells in blood vessels, and also appears to react with cells (possibly pericytes) in capillary walls (Yablonka-Reuveni, personal communication). Another possible candidate is the monoclonal antibody H 36 which has been shown to be specific for rat (but not mouse or human) muscle precursor cells in culture\textsuperscript{102}, although its ability to bind to quiescent satellite cells \textit{in vivo} has not yet been demonstrated. Species differences between mpc are also apparent in the expression of desmin by activated satellite cells. Desmin is present in proliferating rat mpc in cultures examined after 24 h, but not in proliferating bovine mpc\textsuperscript{163}. Although desmin-positive cells are reported in regenerating rat skeletal muscle at 24 h after injury\textsuperscript{87}, it is not yet known whether desmin is present in quiescent rat satellite cells (Allen, personal communication)\textsuperscript{18}. One of the most promising marker for early identification of muscle precursor cells \textit{in vivo} appears to be expression of the skeletal muscle specific genes MyoD1\textsuperscript{199} and myogenin\textsuperscript{216} (Grounds et al., manuscript submitted for publication). Since the muscle precursors (satellite cells) from mature mammalian, avian and amphibian muscle\textsuperscript{66} cannot be definitively identified during the early stages of proliferation, we cannot exclude the possibility that some muscle precursors in muscle regenerating \textit{in vivo} might be derived from other types of mononuclear cells.

There are numerous reports that various cells of mesodermal (particularly fibroblasts and adipocytes) and neuroectodermal origin can give rise to mpc under certain conditions\textsuperscript{68}. It is now known that commitment to myogenesis, and transformation of mesodermal cells such as fibroblasts into mpc, may involve activation of as few as one or two genes out of a family of closely related skeletal muscle specific genes\textsuperscript{19,42,138,199,216}. Of interest in this context are observations that myotubules can form in tissue culture by fusion between myoblasts from dysgenic mice.


\** Another possible way of identifying quiescent and activated satellite cells is the use of viral fluorescent dyes, as these have been elegantly used \textit{in vivo} to demonstrate such cells in frog muscle (Herrera AA, Banner LE. 1990, J Neurocytochem 19: 67-83).
and normal fibroblasts. Changes in extracellular matrix might be involved in vivo in converting mononuclear cells within damaged muscle (other than satellite cells) into MPC. Fully differentiated epithelial cells are transformed into mesenchyme-like cells in the presence of purified collagen, and fibronectin binding can change the phenotypic appearance of myoblasts into fibroblast-like cells. It seems possible that cells of non-muscle origin (particularly mesodermal) might well develop into muscle precursors in vivo (Fig. 1C) in situations where normal muscle tissue architecture and cell communication are severely disrupted: such conditions in regenerating muscle might

Fig. 1. Electron micrographs of presumptive striated muscle precursor cells in regenerating TA muscle. Samples were taken from adult female SJL/J mice 3 days after injury. – A: A satellite cell showing a nucleus containing much heterochromatin. The external lamina (arrow) of the muscle fibre overlies the satellite cell (× 14 200). – B: A mononuclear cell (PM) which may be a muscle precursor but its identity cannot be confirmed in the absence of sarcomeric filamentous arrangements. The cell is contiguous with a myofibre (above) and a macrophage (Mae) (× 9500). – C: A muscle precursor cell identified by the presence of thick and thin filaments arranged as poorly formed sarcomeres (arrow) (× 11 400).
induce the expression of muscle differentiation genes in non-muscle mononuclear cells, and in this way recruit additional muscle precursors.

Satellite cell relationships

It has been shown that satellite cells are part of the same somitically derived myogenic lineage as embryonic muscle cells, and they are often considered to be embryonic precursors trapped beneath the basal lamina of muscle fibres during development. Until recently there was little evidence to deny that satellite cells were not a homogeneous population of cells equivalent to embryonic muscle precursors (reviewed172). However, transplantation studies in chick embryos indicate that postnatal satellite cells are unable to take part in muscle embryogenesis, suggesting that satellite cells represent a differentiated non-totipotential type of myogenic cell31. If it can be demonstrated that the satellite cells had not been immunologically rejected in these experiments, then this strongly supports the idea that there are distinct different populations of mpc in embryos and adult animals. Heterogeneity within populations of embryonic, fetal and adult mpc is supported by the work of Cossu and his colleagues58, who have described differences between mpc derived from early and late embryonic stages, and adult muscles of mice and humans in their response to a tumour promoter36,123, and differences between myotubes formed by mpc derived from mice of different ages in the expression of molecular forms of acetylcholinesterase189 and myosins209. In addition, differences between cultured mpc derived from embryonic and adult chick muscle have been reported in the temporal appearance of desmin (Yablokova-Reuveni Z, Nameroff, M. Temporal differences in desmin expression between myoblasts from embryonic and adult skeletal muscle differentiation, in press) and of three developmental isoforms in fast myosin heavy chains84, and there appears to be an age-related difference in the response of rat mpc to laminin55. Furthermore, centrally located myotube nuclei are conspicuous in vivo in myotubes from mpc of mature muscle, but not in those formed from neonatal mpc274 (Partridge unpublished results).

It is not known whether there are lineages of satellite cells equivalent to the mpc related to primary and secondary myotubes and other myogenic lineages of embryonic muscle179. However, there is recent evidence that different classes of satellite cells may be derived from and reflect the characteristics of muscles of a particular fibre type. For example, slight differences are reported in the production of fast and slow myosins in tissue culture between satellite cells derived from fast and slow chick muscle126. Similarly, specific myosin isoforms are expressed in vivo by newly formed mammalian muscle in orthotopic transplants: this has been shown in transplanted superfascial cat jaw muscle which expresses superfascial myosin in sites innervated by slow or fast motor neurons88, and also in soleus and extensor digitorum longus muscles transplanted into reciprocals sites in denervated legs of mice, where the newly formed muscle cells express either slow or fast myosins respectively, representative of the parent fibre type (Hoh et al., personal communication).

When satellite cells are replicating it is not certain whether the cessation of proliferation and start of fusion is controlled purely by the conditions of their environment186. Alternatively, the switch from replication to fusion of mpc might be dictated by an intrinsic genetic programme which determines the number of cell divisions that must occur before the satellite cell is terminally differentiated and able to fuse, as predicted by the cell lineage model of myogenesis15,162. Autoradiographic studies in regenerating muscles of adult mice70 and chickens72 do not support predictions of the cell lineage model in vivo; however, these results are interpreted on the assumption that satellite cells represent a homogeneous population of muscle precursor stem cells. It is necessary to resolve which model of myogenesis applies to satellite cells, as this issue has important implications for potential in vivo therapeutic manipulation of muscle precursor cell proliferation and fusion after severe injury and in myoblast transfer therapy169,152.

There is very little evidence available to indicate what factors might act either directly or indirectly, in controlling the proliferation and fusion of mpc in vivo, because the situation is clearly very complex. This review will be confined mainly to recent literature on the role of extracellular matrix and growth factors in these processes, and these will first be discussed in general.

Extracellular matrix

Components of the extracellular matrix (ECM) undoubtedly play an important physiological role in maintaining the quiescent state of satellite cells, and in regulating mpc proliferation and fusion175. In addition,
ECM components are involved in the innervation of muscle cells. The ECM surrounding myofibres consists of a basement membrane and associated interstitial connective tissue whose composition is indicated in Fig. 2. The composition of ECM materials interacting with the cell surface has important regulatory and structural consequences: it can affect cell morphology, cytoskeletal organisation, biosynthetic pathways and gene expression, and an extensive literature now documents these roles.

The importance of the basement membranes is demonstrated by tissue culture experiments where the attachment and proliferation of chick embryonic mpc and satellite cells was significantly higher in cells grown on reconstituted basement membrane (containing laminin, collagen type IV, heparan sulphate proteoglycan and entactin) as compared with gelatin (collagen type I). In addition, the differentiation and fusion of mpc were delayed in basement membrane (compared with gelatin cultures) and cultures could be maintained for very long times without fibre detachment and with very strong fibre contractions. Like fibroblasts, mpc can synthesise many of the ECM components during myogenesis and these remain closely associated with the cell membrane.

Laminin is the most abundant glycoprotein in basement membranes. Binding of laminin and fibronectin to cell surfaces can occur through a common receptor, but, in addition, there are specific binding sites for laminin. Since most (but not all) mpc proliferation and fusion during regeneration occur within old basement membranes, laminin is well located to influence this process. An important role for laminin is supported by tissue culture studies of mpc from rodents which show that mpc preferentially adhere to laminin, laminin rapidly stimulates mpc elongation and produces a dramatic increase in motility, and can be produced by mpc.

The response to laminin was pronounced with mpc taken from newborn rats or cell lines derived from adult mice, and was present in cultured mpc from 7- to 19-day-old rodents, but not from 15-day-old rat embryos (where basal laminae cannot be detected). These results support the idea that mpc from different development stages may require and respond to different extracellular environments. Cells such as fibroblasts which are not closely associated with basal lamina, tend to respond in a similar manner to fibronectin rather than to laminin.

Cellular fibronectin is a cell surface and extracellular matrix protein which functions in cell-cell adhesions, cell-substrate adhesions, cell motility, specific binding of macromolecules, and maintenance of a normal morphological phenotype. Cell surface receptors for fibronectin may be glycolipids, glycoproteins or heparan sulphate proteoglycans. Glucocorticoids, growth factors, and cyclic AMP can increase fibronectin levels on cells and extracellular molecules themselves can regulate the quantities of other ECM molecules. Tissue culture studies produce conflicting reports on changes of fibronectin levels during myogenesis: an increase in fibronectin with myofibre formation and subsequent decrease to prefusion levels with myofibre maturation has been described, whereas, in contrast, other studies show that fibronectin is present on the surface of proliferating mpc but is lost from fusing myoblasts and the level of fibronectin mRNAs is drastically reduced after fusion. In support of the latter observations, high levels of fibronectin promote replication and reduce the fusion of mpc from mammalian cell lines. Hyaluronic acid also appears to play an important role in mpc differentiation and fusion as levels of hyaluronic acid in cultured mpc from chick embryos decrease prior to fusion, and in the presence of elevated hyaluronic acid levels fusion is inhibited.

A major component of basal lamina of muscle is heparan sulphate proteoglycan which strongly inhibits mpc proliferation: this is probably an indirect effect and is attributed largely to the strong binding to growth factors like FGF.

One of the earliest ECM components shown to be implicated in myogenesis in tissue culture is collagen, which may be essential for the differentiation and fusion of mpc. Conditions which lead to changes in the forms of collagen secreted by mpc may result in extensive fibrosis and an increase in fibroblast-like cells at the expense of myoblast-like cells.

The interaction of cells with the ECM often involves intermediary ECM molecules like fibronectin, although cells can interact directly with hyaluronic acid and with several types of collagen by plasma membrane binding. The precise relationship between interactions of the ECM components and receptors on mpc (see Section 5), and the role of ECM in (re)innervation (see Section 6) is beginning to emerge.

**In vivo studies.** Corresponding with results from in vitro work, an immunofluorescent in vivo examination of developing chick embryos showed a relative reduction in fibronectin and hyaluronic acid but enrichment of laminin in presumptive myogenic regions at embryonic stage 24 before the onset of myoblast fusion; at the later stages 26 and 28 these patterns were more pronounced. The early appearance of laminin and lower levels of fibronectin and hyaluronic acid in the newly forming basal lamina of developing avian muscle broadly relates to changes in ECM components during muscle regeneration. Immunofluorescent studies carried out in regenerating muscle transplants of adult rats by Gulati show that fibronectin was disappearing from degenerating myofibres by 4 days after autotransplantation, and that laminin, types IV and V collagen and heparan sulphate proteoglycan, although slightly more persistent, also disappeared from old basement membranes. The disappearance of various basement membrane macromolecules is probably due to proteases and other enzymes released by infiltrating cells like macrophages and also from myofibres. However, some components of the basement membranes persist, as shown by binding of Concanavalin A which binds to mannose or glucose subunits of glycoproteins of glycolipids. Laminin was observed in new basement membranes formed around myotubes at day 4, whereas...
fibronectin was the last of the ECM components to reappear\(^{16}\). A similar sequence of fibronectin and laminin disappearance was seen in muscle of regenerating anuran limbs, and fibronectin remained low at the time of mpc fusion\(^{18}\).

In autotransplants of whole EDL grafts in mice, autoradiographic studies have shown that mpc replication starts around 2 days, peaks by 6 days, and is greatly reduced by 9 days\(^{169}\). These combined results indicate that mpc proliferation in such grafts is initiated when significant amounts of laminin and other ECM components are still present in the old basement membranes.

Detailed studies of proteoglycan changes in developing and regenerating muscle have been carried out in chickens\(^{187}\) and rats\(^{86,198}\). In uninjured mature muscle only the small proteoglycans dermatan sulphate and heparan sulphate proteoglycans are synthesised, and heparan sulphate proteoglycans remain relatively high during regeneration\(^{87}\). Developing\(^{27}\) and regenerating\(^{27,86,198}\) muscle synthesise large chondroitin sulphate proteoglycans, one of which is specific to muscle and is rich in 6-sulphate as compared with 4-sulphated disaccharides\(^{27}\) and this form is prominent in mature muscle\(^{86}\). After stretch induced\(^{56}\) or mechanical\(^{195}\) injury in rats there was an increase in unsulphated proteoglycan during the prefusion mpc proliferation stage, and a return to 6-sulphated in the postfusion myotube stage. With respect to these observations, it is interesting that the growth factor transforming growth factor-beta potently increases the expression of chondroitin/dermatan sulphate proteoglycans by muscle precursors and other cells in tissue culture\(^{8}\). These results suggest that large sulphated proteoglycans play a role in some aspect of myogenesis.

Other in vivo immunofluorescent studies have used lectin binding to examine cell surface glycoconjgates in normal and regenerating muscles of adult rats\(^{71,87}\). The observations in normal rat muscle support previous studies in human muscle\(^{153}\) and show a pericellular endomysium rich in α-L-mannose, α-L-glucose, N-acetyl glucosamine, sialic acid, β-D-galactose, and galactose. In transplanted muscles\(^{72}\) a decrease in binding to several lectins in degenerating muscle indicated the breakdown of basement membrane and interstitial connective tissue in these areas. With the onset of regeneration only wheat germ agglutinin (WGA), of the 10 lectins tested, showed extensive binding in the myogenic region (at 4 days). The binding was largely in the extracellular areas and surrounded mpc and myotubes. As the myotubes matured the binding of this and other lectins was restricted to the endomysium and the pattern of lectin binding in 28-day-old grafts was similar but more pronounced than in normal muscle. The specific and extensive binding of WGA in myogenic areas indicates an increase in N-acetylgalcosamine and sialic acids in the ECM during myogenesis, and N-acetylglucosamine has been shown to enhance blast fusion in tissue culture\(^{29}\). Strong staining of mononuclear cells with WGA was also seen in rat muscle regenerating after bupivacaine injury, and these myogenic cells showed similar binding to the lectin Ricinus communis which recognises D-galactosamine\(^{87}\).

**Growth factors**

Growth factors (GF) are peptides which stimulate or inhibit cell division or affect cell differentiation: they are produced locally and often act in an autocrine or paracrine manner. Their mode of action\(^{174}\) and their regulation of protooncogenes during myogenesis\(^{34,43,53,104,180,199}\) will not be discussed here. The effects of GF on the proliferation and differentiation of tissue cultured mpc derived from cell lines, embryonic, and neonatal muscle have been thoroughly reviewed recently by Florini and Magri\(^{32}\). A few studies have also examined the effects of these factors on satellite cells in tissue culture\(^{3,11,37}\) (Fig. 3), although such experiments have rarely been extended to the behaviour of mpc in regenerating adult muscle in vivo. The effects of various hormones and growth factors on developing muscle in vivo has been reviewed recently\(^{155}\).

While it is useful to extrapolate information gained from these tissue culture studies to the situation of mature muscle in vivo, it must be emphasised that conditions in tissue culture are vastly different from the complex environment in vivo. In addition, the rules and conditions that govern the precise arrangement of tissues during development may not apply so rigidly since tissue relationships are fully established, and thus the behaviour of mpc derived from embryonic muscle may be different from that of mpc derived from mature muscle. Furthermore, it seems likely that mpc derived from amphibians, birds or mammals may respond to conditions in slightly different ways.

When investigating the conditions or factors which activate quiescent satellite cells attention must focus on growth factors which are considered to move cells from the quiescent G\(_0\) state into the G\(_1\) phase of the cell cycle\(^{147}\). These GF are often termed competence factors and the most well known are FGF (and its closely related peptide structures\(^{63,64,105,167}\), platelet derived growth factor (PDGF), and macrophage derived growth factor (MDGF): the effects of MDGF overlap with and may be completely equivalent to those of FGF. Other growth factors (termed progression factors) act slightly later in the cell cycle and

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Fig. 3. The main effects of various growth factors and hormones on the proliferation and fusion of mpc are summarised: their action may depend on the dosage and combination with other factors. The information is based on several papers (see text).
regulate the progression through G1 to the DNA synthesis phase: these include insulin-like growth factors (IGFs) and epidermal growth factor (EGF).

**Competence Factors.** Both PDGF and MDGF are attractive candidates for potentially stimulating mpc in vivo, as PDGF would be released by platelets and other cells aggregating in injured blood vessels, and MDGF would be released by macrophages accumulating in the damaged tissue. These factors are closely involved in many aspects of the general tissue repair process. It is well recognised that PDGF is a major mitogen for mesenchymal cells like fibroblasts and smooth muscle cells, and that it induces chemotactic responses in these cells, and there is evidence that PDGF and PDGF-like factors are also chemotactic for embryonic mpc. Studies with crude preparations of platelets in rabbits suggested that platelet-breakdown products stimulate skeletal muscle regeneration and recent data show that PDGF is mitogenic for mpc. Experiments from the laboratory of Jablonka-Reuveni with the C2 cell line (derived from adult mouse satellite cells) show that these mpc have receptors for 3 types of PDGF (BB, AB, AA) and that there is about 10-fold more binding to the BB form. (Jablonka-Reuveni Z; Balestreri TM; Bowen-Pope DF 1990: Regeneration proliferation and differentiation of myoblasts derived from adult mouse skeletal muscle by specific isoforms of PDGF, J Cell Biol, in press). A recent report confirms the selective binding and mitogenic response of rat myoblasts to BB-PDGF. The predominant form of PDGF in rat and mouse serum is the BB type, BB-PDGF (not AA PDGF) enhances the proliferation of mpc about 5-fold, and the effect of BB-PDGF on proliferation and differentiation is similar to that of basic FGF (Jablonka-Reuveni Z, Balestreri TM 1990: Platelet-derived growth factor and fibroblast growth factor promote regeneration of C2 myoblasts to a similar level, manuscript in preparation). The AB form of PDGF has little effect, and as this was used in earlier studies by other investigators Jablonka-Reuveni, personal communication) this may account for the lack of response of satellite cells to PDGF in these experiments: alternatively PDGF may become effective after other factors first activate the quiescent satellite cells. Myoblasts derived from 10-day-old embryos do not exhibit any significant binding of PDGF, although fibroblasts derived from 10-day-old chick embryos do bind the different forms of PDGF (Jablonka-Reuveni et al., 1990: Expression of receptors to platelet-derived growth factors by fibroblasts and myoblast from embryonic chicken skeletal muscle, manuscript in preparation).

The accumulation of mpc at a site of injury is due to local cell proliferation and also to migration of mpc from sites distant to the injury or from adjacent myofibres. The factors (either physical or chemical) resulting from myofibre trauma which attract mpc have not been defined, but may include growth factors such as PDGF.

There are much data which show that basic FGF has a powerful mitogenic influence on mpc in tissue culture (reviewed), and it appears that FGF may play a central role in initiating mpc proliferation in vivo. After muscle trauma, it would be expected that FGF bound to the ECM (specifically heparan sulphate proteoglycans and glycosaminoglycans) surrounding the myofibres would be released by the action of heparanases produced by leukocytes, and by plasmin. FGF would also be produced directly by a range of local cells including macrophages and endothelial cells. Skeletal muscle cells also produce FGF or FGF-related substances. Paradoxically it is not yet known whether receptors for FGF are available on satellite cells. It has been suggested that production of FGF-related oncogene proteins may participate in autocrine and paracrine stimulation of cells. While FGF increases the proliferation of mpc, it also strongly inhibits the differentiation of mpc and appears to act very early in the process of determining whether a mpc will undergo terminal differentiation. Studies in chick embryos indicate a role for FGF in vivo at least in developing muscle. In addition to its effect on proliferation and fusion of mpc FGF may be involved in reinnervation of muscle. However, FGF has wide specificity and stimulates the proliferation of other mesodermal cells like fibroblasts, which may lead to fibrosis after severe tissue damage.

Some factors isolated from skeletal muscle which are mitogenic for mpc have been shown to be either transferin-like or bound in the extracellular space or to be similar to FGF, however, a mitogen specific for mpc does appear to be produced by skeletal muscle and promises to be an important growth factor during muscle regeneration in vivo. This GF is present in uninjured muscle but increases in traumatised muscle and is also a powerful angiogenic agent. It seems to act as a competence factor and its relationship to FGF is unclear.

**Progression Factors.** The insulin-like growth factors (somatomedins) are structurally related peptides, and IGF-1 is usually an order of magnitude more potent than IGF-II. Both IGFs are more potent than insulin and this corresponds to the binding specificity of the IGF-1 receptor. There is ample evidence from tissue culture studies that IGFs powerfully stimulate mpc proliferation and differentiation, and it seems likely that the production of IGF by mpc plays an important role during muscle regeneration. Immunofluorescent studies demonstrate that mpc of EDL muscles regenerating after ischemic necrosis in rats produce IGF-I in satellite cells, nerves, and blood vessels within 24 hours, and IGF-I levels remained elevated for at least two weeks. Subsequent studies showed that IGF-1 mRNA increased at 24 h, peaked strongly at 3 days, and returned towards normal by 10 days. It is very interesting that this pattern of IGF-1 mRNA production in vivo parallels the onset of...
24 h, peak (around 72 h), and duration (around 5 days) of muscle precursor replication in regenerating rodent muscle. The close relationship between replication and production of IGF-I by mpc is further supported by observations with tissue-cultured muscle C2 cells that IGF-I receptors are expressed at their highest level in replicating mpc and decrease by 50% after differentiation. Tissue culture studies show that receptors for IGF are up-regulated by dexamethasone and receptors for IGF (unlike other GF) are not down-regulated during differentiation. In contrast there is a paucity of IGF-II and its receptors in replicating C2 mpc and a rapid increase in differentiating cells and myotubes; this observation supports the proposal that IGF-II may also be involved in mesenchymal conversion. The coordination production of IGFs and their receptors and of IGF binding proteins during myogenesis suggests an autocrine or paracrine role for these GF.

The effects of growth hormone are mediated through IGFs which are bound to specific proteins in serum. It has been demonstrated that growth hormone regulates the local (autocrine) production of IGF in rat skeletal muscle, although tissue culture studies clearly show that IGF can also be produced in the absence of growth hormone by muscle cells. In order to investigate the role of growth hormones in the proliferation of satellite cells from mature muscle in vivo, we carried out studies on lidh mice which have a genetic pituitary dysfunction and lack growth hormone. Preliminary results show no histological differences between new muscle formation after crush injury to tibialis anterior muscles of lidh mice and control C57 B 1/6 J mice (Grounds and McGeech, unpublished data). Similarly, no effect was seen in new muscle formation, or in the production of IGF-1 by regenerating muscle in growth hormone deficient, hypophysectomised adult rats.

A study of muscle regeneration was also carried out in adult rats where serum levels of bound IGFs were elevated by injecting recombinant growth hormone; no morphological differences were noted in muscle regeneration after ischemic injury between rats with elevated IGFs and controls. These in vivo studies indicate that serum levels of IGF do not play a limiting role in regulating satellite cell proliferation during regeneration of mature muscle; however, the (autocrine and paracrine) production of IGF by mpc would seem to be important and is not dependent on growth hormone.

Muscle precursors derived from cell lines and from mouse and human muscle have receptors for EGF, yet they do not proliferate in response to EGF alone; EGF appears to inhibit mpc differentiation, and in muscle cell lines it acts synergistically with FGF to enhance the proliferation of mpc through the G1 phase of the cell cycle. Thrombin has also been shown to act in conjunction with FGF to increase the proliferation and inhibit the differentiation of mpc in tissue culture.

Negative growth factors. Transforming GF beta (TGF-beta) is often considered to be a growth-inhibiting factor, and tissue culture studies show that TGF-beta inhibits the proliferation of mpc (including satellite cells) and is also a potent inhibitor of differentiation and fusion. TGF-beta blocks a step very early in the process of commitment to fusion. There is a virtual disappearance of TGF-beta binding sites on mpc during differentiation and fusion in some cell lines. In isolated myofibres maintained in tissue culture TGF-beta was shown to be a potent inhibitor of satellite cell proliferation. TGF-beta-like peptides are released from many cells in a biologically inactive form which cannot bind to receptors, although whether TGF-beta truly functions as a physiologically negative autocrine or paracrine growth factor in vivo, is still unproven. TGF-beta has effects on many cell types unrelated to the control of proliferation and differentiation. It is released by platelets and is a potent chemoattractant for monocytes, and it also has an extensive role in the formation of ECM: these functions are undoubtedly of major importance with respect to repair after tissue damage. TGF-beta is particularly involved in the rapid formation of collagen (types I, III and V) by fibroblasts within as little as 6 h after injection, and inhibits proteolytic degradation of newly formed ECM. TGF-beta also regulates the expression of fibronectin and related cell surface molecules, and the expression and structure of sulphated proteoglycans which are the major component of ECM in skeletal muscle.

Another potential negative growth factor for skeletal muscle is interferon. This has been shown to inhibit mpc differentiation, but appears far less potent than TGF-beta. Preformed interferons are located within macrophages and polymorphonuclear leukocytes and play a major role in the inflammatory response, and such cells may deliver interferons during muscle regeneration (M. Beilharz, personal communication).

Hormones

In contrast with growth factors, hormones are usually produced in distant sites and arrive via the circulation: the effects of various hormones on skeletal muscle growth have been reviewed.

Adrenocorticotropic hormone. It was proposed that adrenocorticotropic hormone (ACTH) produced by the anterior pituitary might play a role in stimulating mpc proliferation in muscle in vivo, as ACTH has been shown to be a potent and selective mitogen for mpc from both foetal and adult muscle in tissue culture, and is far less mitogenic for fibroblasts. Damage to muscle would result in stress to the animal and a rapid rise in serum ACTH: this probably peaks within one hour and the time taken to return to resting levels depends on the nature of the stress. Melanocyte stimulating hormone is a related neuropeptide derived from pro-opiomelanocortin, and it has a similar potent and selective mitogenic effect on mpc to that seen with ACTH. ACTH-like peptides are widely distributed throughout neural tissues and are also present in non-neural cells such as lymphocytes. It may be that these or related neuropeptides are produced by mpc or myotubes and act locally in a paracrine manner. This appears to be the case in post-implantation mouse embryos.
where ACTH-like peptides are present in myotubes of early limb buds\textsuperscript{38}.

**Glucocorticoids.** Serum ACTH directly regulates the production of glucocorticoids, and the synthetic glucocorticoid Dexamethasone has a strong proliferative effect on immature muscle in tissue culture; like ACTH it is a specific mitogen for mpc but not for fibroblasts. It is proposed that glucocorticoids may play a role in maintaining cell replication in developing muscle in vivo\textsuperscript{7}. The proliferation of satellite cells in tissue culture in the presence of serum has been shown to have a critical requirement for Dexamethasone\textsuperscript{8}. This effect of Dexamethasone on promoting mpc proliferation is dependent upon the presence of other growth factors and may be due to modulation of their effects, as Dexamethasone potentiates factors like FGF\textsuperscript{63}, upregulates receptors for IGF\textsuperscript{213}, and modulates the binding and action of EGF\textsuperscript{9}. Data from two in vivo studies where Dexamethasone was administered either after crush injury to mouse muscles\textsuperscript{18} or transplantation of minced muscle grafts in rats\textsuperscript{196}, provide little support for the idea that this glucocorticoid enhances or prolongs the stimulation of mpc proliferation during regeneration.

**Testosterone.** The response to stress produces complex changes in ACTH, corticosteroids and corticosteroid-binding globulins and may also cause a decrease in plasma testosterone levels (see\textsuperscript{67}). Studies with minced muscle isografts in mice suggested that high testosterone levels can inhibit the phagocytosis and removal of necrotic muscle\textsuperscript{67}. However, other experiments with crush injured tibialis muscles showed excellent muscle regeneration in both male and female SJL/J mice, although slightly more fibrosis was evident in the females\textsuperscript{1}; castration of male SJL/J mice resulted in a histological appearance of regenerated crush lesions similar to that seen for females, and there was no marked difference in autoradiographic data from the 3 groups (Mitchell, Grounds, McGechie, unpublished results). Thus, although testosterone levels may influence the extent of fibrosis, there was no striking effect on mpc proliferation in mature muscle in vivo. However, reports in the literature indicate that testosterone does stimulate satellite cell proliferation in developing muscles of immature animals\textsuperscript{42} and this may be due to altered responsiveness of satellite cells to mitogens\textsuperscript{20}.

**Growth hormone.** It is well recognised that skeletal muscle is a target tissue for growth hormone. It seems likely that growth hormone (somatotropin) has an important physiological role during muscle development in growing animals, although available evidence indicates that it does not play a critical role during myogenesis of mature muscle. Since the effects of growth hormone are mediated through IGFs, see earlier discussion under Growth Factors.

**Replication**

The quiescent state of satellite cells in injured muscle could be due either to (i) factors which actively repress cell replication, or to (ii) the absence of factors which stimulate progression through the cell cycle. There is very little evidence available to indicate what factors might be involved, but certain possibilities will be considered (see Fig. 4).

(i) Elegant experiments by Bischoff on isolated rat myofibres maintained in tissue culture show that the proliferative response of satellite cells to mitogens is depressed in the presence of intact plasma membranes\textsuperscript{13, 14}. This contact inhibition is not due entirely to physical factors but also involves electrical activity, as inhibition of spontaneous or induced contractions by tetrodotoxin increased the proliferative response of satellite cells\textsuperscript{14}. Thus in dead or badly damaged fibres the absence of contractile activity might render the satellite cells more susceptible to mitogen stimulation. Bischoff proposes a sensitive push-pull control of satellite cell quiescence/proliferation which can be highly localised. That this delicate balance is affected by electrical activity is of interest with respect to the activation of a small proportion of satellite cells seen in denervated muscle\textsuperscript{130, 132}. To test the possibility that denervation affects or primes the general population of satellite cells, we autoradiographically compared (in mature BALBc mice) the onset of mpc replication in denervated and innervated muscle regenerating after injury\textsuperscript{132}. The onset of DNA synthesis in mpc responding to myofibre injury was no earlier in denervated muscle, showing that the population of satellite cells was not predisposed to activation more rapidly in conditions which stimulate proliferation. In light of Bischoff's proposal\textsuperscript{14}, it would seem that removal of electrical activity may tip the delicate balance and allow occasional satellite cells to respond to local mitogenic conditions and proliferate. The capacity of satellite cells to respond might also be influenced by their position relative to structures like motor endplates where the microenvironment is different (see Section 6); however, our results\textsuperscript{132} show that most of the satellite cell population remains unaffected by denervation and are in a quiescent state equivalent to that of satellite cells in normal innervated muscle.

It seems highly likely that the balance of different ECM components contributes to the quiescent state in vivo as trauma would rapidly alter the relative composition of the ECM. TGF-\beta might assert an influence indirectly by regulating the molecular structure of the ECM\textsuperscript{8}, although whether TGF-\beta directly acts as a growth inhibiting factor on mpc in uninjured muscle in vivo is not known.

(ii) It has been suggested that various proteases (both Ca\textsuperscript{2+} dependant and independent) produced by macrophages and in the sarcoplasm of injured muscle fibres might be mitogenic to mpc\textsuperscript{134}, but there is no direct evidence to substantiate this. Changes in the relative proportions of ECM components, such as decreased heparan sulphate proteoglycans and increased laminin and fibronectin might contribute to the onset of mpc replication.

Available evidence indicates that in uninjured skeletal muscle the mitogen FGF is normally bound and not available to satellite cells. Similarly, PDGF (and MDGF if indeed it has a mitogenic effect) are not present in
uninjured muscle in large amounts. In addition to a lack of available GF, satellite cells may not be able to bind to these GF as the appropriate GF receptors may not be present on their cell surface: alternatively, the receptors may be present but inactivated in some way. Since Bischoff's muscle GF is extracted from uninjured normal muscle this factor may be normally bound like FGF and unavailable to satellite cells, or satellite cells may not have the appropriate receptors to bind to it, or this GF may act in conjunction with other factors. A transient rise in ACTH after injury might also activate satellite cells.

Our studies of muscle injury in mice show that trauma to muscle fibres (which would result in changes to intracellular calcium levels) is not alone sufficient to induce replication of mpc. In transplants of intact or minced muscle (where the basal lamina is severely disrupted), DNA synthesis by mpc of BALBc mice was not seen before 48 h, yet the same cells can begin DNA synthesis 18 h earlier (by 30 h) after crush injury. It seems likely that the delayed onset of replication is related to the slower revascularisation of injured muscle tissue in the transplants (see Sections 2 and 3). The studies by Bischoff of isolated myofibres in tissue culture also support the idea that satellite cells require the addition of specific GF to start replication.

Once mpc have been activated, the cells move through G1 to DNA synthesis under the influence of various factors including IGF and possibly EGF. Cell proliferation is maintained by elevated levels of combined factors (FGF, IGF, EGF, thrombin, TGF-β), and perhaps hormones such as glucocorticoids in conjunction with multiple changes in levels of ECM components. Many growth factors (e.g., IGF, TGF-β) and ECM components are produced by mpc themselves and may act in an autocrine manner.

5. Differentiation and Fusion of Muscle Precursor Cells in Vivo

Differentiation

At some point during the early part of the G1 phase of a cell cycle, a mpc begins to differentiate and becomes committed to fusion (Fig. 7). Differentiation results in the expression of a range of skeletal muscle genes such as specific muscle regulatory genes, actins, myosins, troponymosins, desmin, and M creatine phosphokinase. Some of these genes are expressed before fusion and others after fusion. Under normal conditions, differentiation of mpc is accompanied by fusion into myotubes (Fig. 5); however, by using agents which block fusion in tissue culture, it has clearly been demonstrated that fusion is not a prerequisite for expression of many of these genes. Furthermore, the induction of muscle specific genes during differentiation can be reversed and mpc made to reenter the cell cycle and synthesise DNA. However, differentiation is a prerequisite for fusion.

Fusion

Many aspects of myoblast fusion, and early work on fusion of cultured myoblasts, are covered in an excellent
review by Bischoff. Fusion between myogenic cells in vivo (in regenerating mature murine muscle) is the subject of an extensive ultrastructural study recently completed in our laboratory (Robertson TA et al. – manuscripts submitted for publ.).

Fusion is a highly complex phenomenon concerning a whole range of biochemical and biophysical interactions. The barriers to fusion, particularly electrostatic repulsion due to glycoproteins and glycolipids, must be overcome. Cytoskeletal elements can control the movement of certain integral membrane glycoproteins out of an area where membranes are about to fuse (Fig. 6), while other proteins can be moved into the area to form junctions for fusion.

Reorganisation of the cytoskeleton and redistribution of plasma membranes proteins has been shown in prefusion mpc. Elevated Ca++ levels and H+ (decreased pH) are implicated in many ways in inducing fusion of membranes. Cations like Ca++ can neutralise negatively charged membranes and reduce their electrostatic repulsions, and may be involved in expression of calcium dependent proteins in modifying the cytoskeleton. Membrane fusion is specifically dependent upon external calcium, and the rise in intracellular Ca++ levels essential for mpc fusion appears to be mediated by Ca++ dependent protein kinases. For cells to fuse together it is necessary for adhesion specific receptor-receptor or
receptor-site bonds to be formed, and thus expression of specific cell surface receptor proteins is fundamental to the process. Many changes have been seen in cell surface proteins of cultured mpc at the time of fusion\(^1,177\). The alignment of mononuclear mpc and their fusion, either together or with the ends of damaged myofibres, must require cell recognition, neutralisation of electrostatic forces, and the production of specific membrane glycoproteins and glycolipids. The role of ECM, receptors and growth factors will be briefly discussed, but no attempt will be made to examine the role of calcium and other factors influencing this process (see Fig. 7).

Extracellular matrix

It is difficult to comment upon the probable influence of different components of the ECM during myoblast differentiation and fusion \textit{in vivo}, although they undoubtedly play an essential role (see Fig. 7). Available evidence does indicate that decreased amounts of hyaluronic acid and fibronectin may be a prerequisite for fusion, and that increased collagen and N-acetylglycosamine and sialic acid may enhance fusion.

Cell surface molecules

Specific changes in cell surface molecules during myogenesis have been documented\(^1,188,212\) and some of these cell surface molecules have been characterized. The expression of various receptors is undoubtedly fundamental to the specific self recognition of mpc, their interaction with ECM, and their alignment and fusion, but at this stage there is little information on which receptors are implicated. The integrin receptors are a family of transmem-

Fig. 6. Electron micrograph showing fusion between two myotubes. The apposed plasmalemmal membranes of the two myotubes (hollow arrows) are discontinuous and absent in areas where the sarcoplasm of the two myotubes is continuous (asterisks). TA muscle from adult female SJL/J mouse sampled 4 days after injury (× 21 300).
brane glycoproteins which interact with ECM glycoproteins (such as laminin, fibronectin and vitronectin), complement, and other cells, while their intracellular domain interacts with the cytoskeleton. The presence of integrin-like receptors appears essential for fusion, as it has been shown that antibodies which bind to these receptors block differentiation and fusion. In addition, synthetic peptides which bind to the integrin receptors greatly stimulate the Ca²⁺-independent aggregation of mpc, and there is evidence that glycoproteins are important for cell recognition and fusion. The major histocompatibility class I antigens are expressed on myoblasts, disappear after fusion and are absent from myotubes, and fusion is inhibited by antibodies to these (and Thy-1) antigens. These antigens are transiently expressed in regenerating muscle, and also appear to play a role in the fusion of mpc.

It is known that cell adhesion systems can operate either dependent or independent of calcium. The calcium-dependent cell adhesion molecule N-cadherin is found primarily in nerve and muscle, and analogues of N-CAM are present in muscle. Of the calcium-independent cell adhesion molecules, the best characterised is neural-cell adhesion molecule (N-CAM) which (like Thy-1) has structural components in common with immunoglobulins. N-CAMs are cell surface sialoglycoproteins and a diversity of N-CAM isoforms are apparent during development and in different cell types; changes in N-CAM and mRNA accompany the various stages of myogenesis and are seen after denervation or injury of adult muscle. Isoforms of N-CAM specific for skeletal muscle have been identified and are found predominantly on multinucleated myotubes; however, the role of N-CAM isoforms and other CAM molecules in mpc migration, proliferation and fusion, and myotube-neurone interactions remains to be elucidated. The calcium dependent-phosphorylation of a 48 kDa cell-surface protein does appear to be required for fusion.

In injured muscle the binding of the lectin Concanavalin A indicates the presence of mannose or glucose subunits in glycoproteins or glycolipids in degenerated basal lamina in vivo. Concanavalin A binding sites are distributed uniformly in undifferentiated mpc in tissue culture but have a patchy pattern in differentiated mpc and myotubes. It has been suggested that cell surface components rich in these mannose-type of glycoprotein glycans are essential for mpc differentiation and fusion, although this has been disputed.

Studies using various muscle cell lines grown in tissue culture show that receptors for FGF, EGF and TGF-β are down regulated during differentiation, and this may be triggered by a depletion of specific mitogens. While down regulation of these receptors may be a prerequisite for fusion it does not seem to represent a commitment.

Growth factors and prostaglandins

It seems highly probable that fusion of mpc in vivo may be retarded or enhanced by the relative levels of different growth factors (see Figs. 3, 7). The mitogens FGF and PDGF are potent inhibitors of mpc differentiation and thus high levels of these GF would lead to an accumulation of proliferating mononuclear mpc. This population of mpc would be further maintained by high levels of TGF-β. Recent evidence indicates that the inhibition of myogenic differentiation by FGF and TGF-β (and by oncogenes) involves the down regulation of MyoD1 gene expression (EMBO workshop on Cell Mol Biol Muscle Regen, September 1989, Cambridge, UK). In contrast with these GF the mitogen IGF stimulates differentiation and fusion. A decrease in available FGF and TGF-β combined with an increase in cell density and associated increase in IGF (produced by mpc), would favour the differentiation and fusion of mpc. TGF-β is intimately associated with moderating and stabilising the formation of EC (ECM), and in this way TGF-β may be further implicated in regulating mpc fusion in vivo.

Changes in cell membranes necessary for fusion probably also involve prostaglandins which are a family of compounds derived from essential fatty acids such as linoleic acid. It has been proposed that the last major membrane reorganisation before bilayer fusion of mpc is dependent upon the binding of prostaglandin (possibly prostacyclin) to its receptor. Prostaglandins are closely associated with changes in cyclic AMP, activation of protein kinase C, turnover of inositol phospholipid and intracellular Ca²⁺ levels. Increased levels of prostaglandin E₂ (PGE₂) precipitate cell fusion in embryonic chick myoblasts grown in tissue culture, and inhibitors of prostaglandin synthesis prevent fusion. Furthermore, embryonic avian myoblasts synthesise PGE₁ and appropriate receptors, and release PGE₁ into the culture medium resulting in fusion. These and other studies all indicate that changes in prostaglandin levels are central to cell fusion, and that the function of prostaglandins may be to trigger cell fusion once the mpc density and associated (autocrine) production of PGE₁ is sufficiently high; however, some studies have failed to confirm the observations of Zalun. The role of prostaglandins was investigated in vivo during muscle formation in chick embryos and, while administration of PGE₁ reduced the number of myonuclei, incorporation into myotubes confirming predictions of in vivo studies, it is unclear why inhibitors of prostaglandin synthesis resulted in myopathy and smaller muscles. Further studies by McLennan have evaluated the role of various prostaglandins and related substances on developing muscle in chick embryos in vivo. There is no information about the role of prostaglandins on myogenesis of mature muscle in vivo, although a relationship between increased mpc density and associated increased levels of prostaglandin contributing to cell fusion is attractive.

6. Myotubes and Innervation

This review is concerned mainly with the proliferation and fusion of mpc. There is a vast literature relating to ensuing events in myotubes, but these important aspects (fundamental to the function of skeletal muscle) will only
be discussed briefly. After mpc have fused to form multinucleated myotubes and contractile myofilaments are assembled, the expression of various genes (like actin, myosin and tubulin) changes often from embryonic or juvenile isoforms to the adult form. Successful formation of synapses and motor endplates connecting the muscles and contractile apparatus to appropriate motoneurones completes the maturation of the new muscle cells.

Muscle injury may result in both damage to nerves and removal of feedback from muscle synapses to specific nerves. These events can result in the release of various agents which stimulate the sprouting of nerve endings and can thus aid reinnervation of regenerated myofibres (see later). In addition it is possible that myotubes themselves produce neurotrophic factors. There is good evidence from studies of developing embryonic muscle that skeletal muscles produce specific molecules such as FGF. which act on motoneurones to promote axonal growth, and it is proposed that IGF-II may be produced by myotubes and stimulate neurite growth and synapse formation; although the extent to which these apply to regenerating muscle is unknown.

Before nerves make synaptic contact with new muscle cells, surface molecules like N-CAM and acetylcholine receptors (Achr) are distributed relatively evenly over the cell surface. When a synapse is formed N-CAM and AchR cluster and become restricted to synaptic sites. Recent research has identified many factors such as calcitonin-gene related protein which regulate AchR. and these probably play a role in synapse formation in vivo (review). Where new myotubes are formed within old basement membranes, AchR clustering is seen on the surface of the myotubes underlying and contacting the old synaptic sites even in the absence of axon terminals. AchR clustering at new synaptic sites and in myotubes beneath old synaptic sites appears to be due (at least in part) to proteins such as agrin and gelatin. Antibody studies show that agrin is concentrated in the synaptic basal lamina at the neuromuscular junction in vivo in a range of species and is probably secreted by nerve terminals of motoneurones. Agrin (or an agrin-like molecule) can also be produced by muscle in the absence of nerves in developing aneurial chick muscles. The presence of the glycoprotein gelatin in synaptic areas.

**Fig. 7.** Summary diagram to indicate factors which may [(i)] maintain mpc replication by stimulating proliferation and/or by inhibiting the differentiation of these cells, and [(ii)] conditions which favour cessation of proliferation and fusion of mpc.
correlates with successful reinnervation, and gelasmin appears to be produced by muscle rather than the nerve. When the muscle cell is innervated, muscle nuclei and Golgi apparatus become immobilised beneath the synapse: the immobilisation involves microtubules, actin filaments and perhaps tropomyosin. These junctional nuclei are transcriptionally different and synthesise more AchR than nuclei not localised beneath the synapse, and the junctional synapse area almost resembles a minicell.

The precise way in which motoneurones make connections with new basement membranes of myotubes depends in part upon whether the myotubes lie outside or inside the scaffolding of a persisting old basement membrane. Where a myotube has been formed outside the persisting basement membrane of injured fibres, as may happen after severe injury or in minced muscle grafts, the new myofibre is innervated de novo. The formation of such synapses probably occurs in a manner analogous to the formation of neural connections in developing muscles although at a much later stage of myotube development. Similarly, functional endplates form in ectopic locations on regenerated "whole" muscle fibres of transplants which lack the zone of original endplates.

In most situations in muscle regenerating after injury, or transplantation of intact myofibres, the myotubes lie within an old basement membrane, and the regenerating axons are attracted back to the old synaptic site where the original muscle fibre was innervated. In addition to AChR, N-CAM, agrin and gelasmin, synapses have been shown to have concentrations of particular substances. A synaptic-specific carbohydrate has been identified in vertebrate muscle which resembles N-acetyl-d-galactosamine and is associated with acetylcholine esterase and glycolipids, and a homologue of laminin which is known to be adhesive for motoneurones, s-laminin, is concentrated in basal lamina at synaptic sites. Recent research has shown that fibroblasts proliferate in perisynaptic regions after denervation of rat skeletal muscle in vivo, and it appears that these fibroblasts manufacture cell adhesion molecules like N-CAM, fibronectin, heparin sulphate proteoglycan, and the glycoprotein tenascin which accumulate in these areas. These four adhesive macromolecules are known to interact with neurones, and the coordinated increase and selective perisynaptic localisation after denervation suggests that ECM around old synapses may provide an attractive substrate for regenerating axons.

It will clearly be difficult to identify which of the many interacting factors play a critical role in controlling myogenesis of mature muscle in vivo, but some of the major influences are tentatively summarised in Figs. 4 and 7.

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Abbreviations

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<tr>
<th>Abbreviation</th>
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<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<td>EGF</td>
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<td>Fibroblast growth factor</td>
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<td>Insulin growth factor</td>
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<td>MDGF</td>
<td>Macrophage-derived growth factor</td>
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<td>mpc</td>
<td>Muscle precursors cell(s)</td>
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<td>N-CAM</td>
<td>Neural cell adhesion molecule</td>
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<td>PDGF</td>
<td>Platelet-derived growth factor</td>
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<td>TGF-β</td>
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<td>WGA</td>
<td>Wheat germ agglutinin</td>
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