Differentiation of Skeletal Muscle Cells in Culture

Nibaldo C. Inestrosa

Laboratory of Neurophysiology, Department of Cell Biology, Catholic University,
P.O. Box 114-D, Santiago, Chile

Abstract. The current knowledge of skeletal muscle cultures and the molecular events taking place during muscle differentiation in vitro are reviewed. Skeletal muscle cells differentiate in a unique sequence. Mononucleated precursor cells (myoblasts), which are obtained either from embryonic muscle tissues (primary muscle cultures) or from muscle cell lines, divide exponentially in culture. After cessation of division, myoblasts start to fuse forming multinucleate myotubes. Subsequently, the myotubes develop myofilaments and cross-striations. In this review the concept of fusion is discussed in relation

Abbreviations: ACh, acetylcholine; AChE, acetylcholine esterase; T-system, tubular system; SR, sarcoplasmic reticulum; LC, light chain.
to its measurement and specificity. The changes in the levels of surface (acetylcholine receptors) and cytoplasmic proteins (myosin) are documented. Finally, the differentiation of skeletal muscle cells from neuroectoderm derivatives, and recent tissue culture studies on human skeletal muscle are discussed.

I. Introduction

Muscle cells are useful for studies of cellular differentiation because morphological and functional changes occurring during myogenesis are well characterized and can be observed in tissue culture. The changes that take place during the development of skeletal muscle are among the most dramatic in the entire body. Skeletal muscle arises from small undifferentiated mesodermic uninucleate cells in the embryo and forms large multinucleated cells that are specialized to perform the wide range of movements required by the adult animal. Myogenesis is an attractive model for biologists interested in differentiation mechanisms because the product of muscle differentiation, the myotube, is easily recognized. The sequence of events underlying the final stages of myogenesis is of considerable interest in itself, yet it may also serve as a model for studying terminal differentiation (99). The present review concerns with the formation of skeletal muscle in tissue culture.

Differentiation in general is marked by myoblast proliferation to confluence, followed by progressive fusion to form large multinucleated syncytia (myotubes) that synthesize muscle-specific proteins and contract spontaneously. Before considering certain aspects of myogenesis in more detail I will first define some terms, describe the culture system, and give an overview of the developmental process. Cells employed in most of the experiments are taken from fetal or newborn rat leg, or from embryonic chick or quail skeletal muscle. Usually, the chick embryos used are ten to twelve days of age, the quail embryos are nine days old, and rat embryos are nineteen days old. At these stages of development, several types of cells are present in the dissected muscle tissue, including fibroblasts, presumptive myoblasts, myoblasts, myotubes and myofibers. Fibroblasts are proliferating cells that form connective tissue. A presumptive myoblast is the mononucleated cell which does not fuse or synthesize myosin. A myoblast is the mononucleated cell capable of fusion and synthesis of contractile proteins; the myoblast is the true precursor of the muscle cell, it may divide and form more myoblasts, or it may fuse with others into a myotube. Myotubes are multinucleated cells with centrally-located nuclei and peripherally distributed myofibrils. Sometimes the terms myotube and myofiber are used interchangeably, but if a distinction were to be made, a myofiber should be the most mature form of a myotube.

II. Different systems

a) Primary cultures. Tissue is prepared for culture by dissociating it enzymatically and mechanically into a suspension of mononucleated cells and myotube fragments. Single cells are removed by filtration and plated at a known density on Petri dishes. Culture media vary widely in composition, but each recipe generally calls for a base of Eagle’s Minimal Essential Medium or Ham’s F-10 supplemented with horse serum and chick embryo extract. One of the traditional methods for selecting myoblasts as opposed to fibroblasts is the pre-plating technique of Yaffe (143). This is based on a
greater differential adhesion of fibroblasts when plated for half-hour on uncoated plates. During the first few hours after plating, about half of the cells attach to the substrate and elongate while the rest die. Some cells fuse during this early period, but the majority divide by the end of the first day in culture. After one or more rounds of replication, depending on culture conditions, most of the myoblasts line up into chains and fuse with one another. Under favorable conditions, most fusion occurs within a single ten-hour burst (94). While a few mononucleated myoblasts may show cross-striations and other overt signs of differentiation (32), the majority of muscle cells fuse before initiating synthesis of contractile proteins (98) and other cytoplasmic constituents (117). Receptors for acetylcholine (ACh) may appear on mononucleated cells (121), but the density of these membrane proteins rises usually after fusion (49). Myofibrils fill the cytoplasm over the next several days, accompanied by development of the sarcomere system (39). The simultaneous occurrence of myofibril formation and membrane proliferation is considered to be important in understanding the coordinated events resulting in the differentiated myotube (60). By one week in culture, in vitro myofibers closely resemble their in vivo counterparts, save for branching and hypernucleation. Fibroblasts continue to proliferate during this time, and overgrow the culture if left unchecked.

Morphologically and ultrastructurally, muscle cells are characterized by an elongated shape, hypernucleation, cross-striation, and the sarcomere system. In developing chick muscle cultures Ezerman and Ishikawa (30) have shown that the transverse tubular system (T-system) can be clearly distinguished from the sarcoplasmic reticulum (SR). Both membranous systems proliferated simultaneously at the earliest myotube stage. Electron microscopic observations suggested that SR membranes develop from the rough-surfaced endoplasmic reticulum as tubular projections. The T-system tubules were formed by invagination of the sarcolemma (30). Chick muscle cells often show elaborate three-dimensional networks of a membranous system. The network consists of tubular units which are quite regularly arranged. The tubular units composing the network are accessible to ferritin particles suspended in the culture medium, suggesting continuity with the extracellular fluid (60). The development of the SR and the T-system is also coordinated during postnatal differentiation of rat skeletal muscle (113).

The basal lamina is apparent only in older cultures, which is possibly related to the inability of older myotubes to fuse with myoblasts (39). Mitochondria also show a characteristic pattern of development. In myoblast and nascent myotubes these organelles are short and ovoid, with few cristae; in more mature myofibers, mitochondria become elongated with dense parallel cristae (39). Corresponding temporally with this ultrastructural development there is a metabolic change. Myoblasts are resistant to antimycin A and oxygen deprivation, while multinucleate myotubes are sensitive to both (105). The properties of the surface membranes are also different in myotubes and myoblasts. Myotubes are more sensitive to saponin treatment (91). This has been shown by protein leakage studies as well as electron microscopic investigation using ferritin particles, which penetrate in most large myotubes but not into mononucleated cells (91).

b) Clonal analysis. In a variant of the procedure previously described, dissociated cells can be plated at a very low “clonal” density so that each survivor produces an isolated colony (46, 71). After five or six days myotubes form in some of the colonies, while other clones appear to contain fibroblasts only. Myotubes develop in these
clonal cultures much as they do when cells are plated at higher densities, except for a time lag in the former case. Konigsberg (71) has given the best evidence for a morphological distinction between myoblasts and fibroblasts. He described two cell types present 12 to 18 hours after plating embryonic chick cells at clonal densities: (a) a bipolar cell-type with a small ruffled membrane, usually on one tip, and (b) an extremely flattened cell-type with an extensive, unlocalized, ruffled membrane. The former yields a muscle colony, and the latter produces a "fibroblastic" colony, i.e., one without myotubes. The reliability of this technique is fairly high, but not perfect. Analysis of early myogenesis is difficult, because of the asynchronous development in situ. By removing single cells from developing muscle and growing them as clones in culture, cells with the ability to from muscle can be distinguished from cells that give rise to nonfusing cultures. Using this technique the change in numbers of muscle precursor cells with development has been studied in chick (13) and human (44) limb muscle. Clonable myoblasts appear in human limb on about day 33 of development and in chick limb on about day 3. In human leg muscle there is a 6- to 7-fold increase in muscle-forming cells between 5-14 weeks, while a similar change takes place in only 6 days in chick leg muscle. Based on differences in the appearance of muscle clones from early and late fetuses, Hauschka (45) proposed the existence of 'early myoblasts' which give rise to either myotubes or differentiate into 'late myoblasts'. A close relationship between 'early' and 'late' myoblasts is suggested because manipulations of the culture media can provoke 'early' myoblasts to give rise to myotubes clones identical in morphology to those derived from 'late' myoblasts taken from older fetuses.

Several studies suggest that the myoblast's potential for gene expression changes through a maturation sequence that is dependent on innervation. For example, it has been shown in chick muscle, using the clonal analysis that both the ability of myoblasts to fuse in vitro, and the morphology of myotubes, appear to be regulated by neural contact within the limb bud (11, 12). Similarly, Koenig and Vigny (70) observed that myotubes in primary rat muscle cultures could make 16S acetylcholinesterase (AChE) in the absence of nerves only if derived from embryonic muscles that had already been innervated; myotubes formed by cells from uninnervated muscles could make 16S AChE only if co-cultured with nerves. However, we have shown that mouse clonal skeletal muscle cells cultured in vitro can synthesize AChE with a sedimentation coefficient of 16S in the absence of neural influence (59,120, see also 125). Because the clonal muscle cells were derived from limb muscles already innervated by motor nerves, they probably represent an advanced stage of myoblast differentiation that results from in vivo exposure to neuronal influence before isolation of the myoblasts. Sugiyama (125) has contended that it is unlikely that the myoblasts (precursors of the G8-1 cell line) are innervated directly. He argued that the synthesis of the 16S AChE is not dependent on innervation, and therefore the neuron-muscle interaction may not necessarily be the specific factor in the control of the 16S AChE. Some recent experiments have led to the suggestion that muscle contraction activity is the most essential factor in the appearance of 16S AChE (107, 112). However, studies in non-innervated rat muscles in utero (47), as well as in mouse embryos homozygous for muscular dysgenesis (108), have indicated that the 16S AChE appears in the absence of muscle contractions. Our own observations in C2 mouse clonal cells showed that the 16S AChE is not specifically regulated by muscle activity (56, 57).
III. Molecular events taking place during myogenesis

The temporal sequence of several molecular events taking place in the nucleus and cytoplasm of differentiating myoblasts is known. Before myotube fusion, the in vitro activity of the DNA polymerase decreases, DNA synthesis ends, and cellular division stops (95, 102). The concentration of cyclic AMP increases and that of cyclic GMP decreases transiently (83, 84, 151), immediately before the rapid rise of the enzyme activities peculiar to muscle cells: creatine phosphokinase, glycogen phosphorylase, and adenylate kinase (20, 117). At this stage, the contractile proteins actin, myosin, and tropomyosin become detectable (98, 146), as well as the first morphological features of differentiation: thin and thick filaments become observable with electron microscope (53). In differentiated cells, the synthesis of messenger RNA for contractile proteins increases progressively until the levels measured in skeletal muscles fibers are reached (54, 97, 124). Myoblast maturation in vitro is accompanied by structural and functional modifications of the cell surface, such as: the appearance of myotube-specific antigens (134), ACh receptors (57, 128), junctional ACh receptors with an isoelectric point slightly lower than that of extrajunctional receptors in mouse, but not in chick myotubes (126, 127); clusters of ACh receptors (57, 128), AChE (49, 57), the endplate form of AChE (56, 58, 125), clusters of 16S AChE (58), and synaptic basal lamina antigens (59, 120). The amount of fibronectin is greatly increased in the passage from myoblasts to myotubes (55) and its pattern changes from a fibrillar network to a cluster distribution (18). Finally, infolding of the T-tubule system takes place (60) and the cells become able to generate action potentials through the membrane (57, 64).

IV. Fusion

In general, myoblast cultures go through three morphologically and biochemically distinct developmental stages. The myoblasts initially proliferate, proceeding through at least one round of cell division, and are quite motile compared to other cultured cells (145). In the second phase of development cell division ceases but migration continues, this time leading to the alignment of single myoblasts in long chains. Alignment is followed rapidly by membrane fusion and the formation of the multinucleate myotube. Myoblast fusion is the first major step on the path to functionally mature muscle. The potential muscle fiber must next develop the contractile machinery itself and the necessary structures for conduction of the excitation signal from the surface ACh receptors to the contractile proteins. The most striking feature of a skeletal muscle culture is the rapid formation of long, multinucleated syncytia from a layer of mononucleated cells. Since the overall course of fusion is so easily observed with low-power light microscopy, it is the most commonly used marker of muscle differentiation. Fusion is a relatively rapid event, requiring less than one hour. It involves cell recognition and membrane-membrane interactions (7).

The fusion of mononucleate myoblasts is almost certainly a multistep process, including at least the following separable components: (1) cell migration, recognition, and alignment, and (2) membrane fusion leading to cytoplasmic continuity (67, 86). Ultrastructural studies have suggested that fusion is the result of the partial disappearance of the plasma membrane between two adjoining cells (63, 75, 118). An increase in membrane fluidity precedes myoblast fusion (51, 104), and perturbations which are expected to alter fluidity, alter the recognition and fusion processes (68,
Kalderon and Gilula (63) have proposed a model for myoblast membrane fusion based on ultrastructural analyses in which they suggested that cytoplasmic, unilamellar, particle-free vesicles closely apposed to the plasma membrane, fuse with the plasma membrane. This process generates particle-free plasma membrane domains which they suggest are an essential component of the fusion process, i.e., that the particle-free bilayers of two adjacent myoblasts could fuse. David et al. (25) have suggested that an increase in concentration of intracellular calcium triggers the fusion of these cytoplasmic particle-depleted vesicles with the overlying plasma membrane in the same manner as calcium triggers exocytosis of secretory vesicles in other cells (72).

a) Measurement. The usefulness of fusion as an indicator of muscle differentiation depends critically upon both its ease of measurement, and its specificity to muscle tissue. In reference to the former, fusion is quantified simply by selecting random fields under a light microscope and scoring the fraction of nuclei in multinucleate cells. It is very difficult, however, to establish the precise time of fusion by use of the light microscope. Rash and Fambrough (106) fixed the point of fusion electrophysiologically by testing for the appearance of high-efficiency electrical coupling between pairs of cells. Immediately after fusion the cultures were fixed and tilting analysis was performed on serial sections in an electron microscope. They found evidence for the formation of gap junctions between cells and calculated that the actual fusion process could be very rapid, with surface membranes disappearing at a rate greater than 1 \( \mu \text{m}^2/\text{sec} \). The cytoplasmic contents of the multinucleated cell were often poorly mixed shortly after fusion, suggesting slow diffusion of myoblast contents into myotube.

b) Specificity. The reliability of fusion as a muscle marker has been investigated autoradiographically by Yaffe and Feldman (147). These workers have shown that \([^{3}\text{H}]\)-thymidine-labelled heterologous cell populations (liver, kidney, chondrocyte) are not incorporated into multinucleated cells when added to pre-fusion or actively fusing cultures. Some experiments have suggested the possibility that cardiac myoblasts may sometimes fuse into muscle fiber of skeletal origin (144). Accordingly, it appears that a myoblast will fuse only with other muscle cells, and not with any cell that happens to be in its immediate vicinity. It is remarkable that myoblasts from entirely different animal species can fuse with each other if they are at the right stage of development (142, 147). For example, if chick myoblasts are mixed with rat myoblasts, myotubes containing both rat and chick nuclei are formed. The proportion of chick nuclei in the myotubes will in general depend on the ratio of chick to rat myoblasts in the mixed culture. In these hybrid myotubes, myofibrils containing both chick and rat muscle proteins are formed, and similarly the proportion of chick proteins is a function of the ratio of chick to rat nuclei in the particular cell (17).

The manner in which the nucleus and cytoplasm interact to regulate the expression of specific functional activities of cells is a central problem in Cell Biology. In particular, it can be asked whether a myoblast nucleus retains its program for myogenic differentiation after a period of residence in the cytoplasm of a non-myogenic cell. Ringertz et al. (109) used rat myoblasts and mouse fibroblasts enucleated by centrifugaton of cytochalasin-treated monolayers, to form intact cells by fusion of nuclei ('minicells') from enucleated rat myoblasts and cytoplasms from enucleated mouse fibroblasts. Clones derived from reconstituted cells formed myotubes that produced myosin and developed the cross-striated pattern typical of skeletal muscle. Thus, the myogenic program of the rat myoblasts persists through the enucleation and
reconstitution procedures, and it is not altered by a period of exposure to mouse fibroblast cytoplasm.

A marker closely related to fusion is the lining-up of myoblasts into chains. Significantly, non-fusing heterologous cell types do not line up with myoblasts, indicating that a recognition process is involved in this type of aggregation (147).

c) Differentiation without fusion. Two factors that affect the ability of myoblasts to fuse should be mentioned. The first is the finding of Shainberg et al. (116) that unless the culture medium contains sufficient calcium, fusion does not occur. In fact both fusion and lining-up are inhibited in media with low levels of calcium (25 μM) and EGTA (1.9 mM) (98, 116). When cultured in these media, myoblasts divide one or more times (but do not proliferate indefinitely) and eventually assume a long (up to 1 mm) spindly shape. The morphology of fibroblasts is apparently unaffected. Upon addition of 1.4 mM calcium to the blocked cultures, fusion is initiated within four hours and occurs so rapidly that the process is virtually complete within twelve hours. The rapidity of fusion upon calcium release has been interpreted as if these treatments prevent only the activation of the fusion mechanism and not the synthesis of its components.

d) Aging. A second factor that affects the ability of myoblasts to fuse is the age of the myogenic culture. Bischoff and Holtzer (8) presented evidence suggesting that both myoblasts and myotubes from old primary cultures have greatly reduced capability for further fusion.

Lastly, because fused cells never divide (93), the postmitotic condition of myotube nuclei is another indicator of the differentiated state. Holtzer has argued for a withdrawal from the cell cycle prior to fusion (8). Though it is clear that some myoblasts are postmitotic (G1 phase) there is no evidence for an obligatory withdrawal prior to fusion (14, 94).

V. Differentiation of myosin and acetylcholine receptors, two of the most characteristics macromolecules of the skeletal muscle cells

I will now describe some of the characteristics and developmental changes of two well known macromolecules of skeletal muscle cells: myosin, a cytoplasmic protein related to the contraction of the muscle, and the ACh receptor, a membrane protein responsible of the transduction of chemical (ACh) into electrical information (action potential).

a) Myosin. The synthesis of myosin and actin, with subsequent formation of myofibrils, is one of the most characteristic features of a muscle cell. While actin is found in many cell types (102), including fibroblasts, skeletal muscle myosin has unique properties that distinguishes it from other myosins (102). Consequently, it would be expected that skeletal muscle myosin is a useful marker of the differentiated myotube.

A number of techniques have been used to assay myosin production, or alternatively, myofibril assembly. These methods include direct myosin extraction (98), immunological probes (92), electron microscope identification of thick (150 A) filaments (39), and observation of myofibrillar birefringence with polarization optics (93). All of these techniques yield similar results, though their sensitivities vary.

In both rat (146) and chick (98) muscle cultures, the rate of myosin synthesis increases rapidly four to six hours after fusion and remains high for several days.
In EGTA-fusion blocked cultures the rate of myosin synthesis remains low until calcium release, where upon it rises 8 to 10 hours after the onset of fusion. The rate of actin production in muscle cells shows a similar fusion dependence (97). It has been shown that myosin is synthesized by skeletal muscle cells only after they have ceased dividing. As noted earlier, nuclei never divide within myotubes, and it is clear that all myotubes synthesize myosin.

As discussed above, the synthesis of contractile proteins becomes prominent at, or slightly after, the onset of fusion. Upon reflecting on this observation, one question comes to mind concerning the messenger RNA’s that specify these proteins: Are these templates synthesized immediately prior to synthesis of the corresponding proteins, or does transcription occur at an earlier stage of development? Buckingham et al. (15), using fetal calf muscle cultures, reported that the level of 26S mRNA that directs the synthesis of the heavy chain of myosin in a cell-free system, rises 4 to 8 hours before fusion, but becomes associated with the heavier polysomes only after fusion. Furthermore, these same workers reported that this mRNA is not found in mouse fibroblasts or bovine liver, but it is present in pre-fusion cultures. The half-life of the presumed myosin mRNA is only about 10 hours in myoblasts, but increases to over 50 hours in myotubes. Accordingly, it is possible that terminal differentiation, as expressed by heavy-chain myosin synthesis, is affected by mRNA stabilization, and not by initiation of specific mRNA synthesis. Whalen et al. (136) have shown that the heavy chain found in the fetal rat is unique to the embryo and distinct from adult fast and slow heavy chains of myosin. On the other hand, Masaki and Yoshizaki (76) demonstrated that chicken embryonic skeletal muscle myosin reacts not only with anti-fast myosin antibody but also with anti-slow as well as anti-cardiac myosin antibodies. Gauthier et al. (43), studying rat diaphragm muscle with antibodies against chicken myosins, showed that intense staining of all fibers occur with both anti-fast and anti-slow myosin antibodies at 19 days of gestation. One day after birth fiber type distinctions were already evident. Therefore, it is possible that embryonic tissue contains fast, slow, and embryonic types of myosin. Recent studies of Whalen et al. (137), using various approaches including polypeptide mapping, complement fixation, immunocytochemistry, gel electrophoresis of native myosins, and the study of synthetic myosin thick filaments by electron microscopy, have clearly shown that three different heavy chain isozymes of myosin appear sequentially in rat muscle in the period between late gestation and about three weeks of age. In part these changes can be related to changes in innervation taking place during this period. The above results indicate that the predominant myosin heavy chain isozymes in developing muscle are neither adult fast myosin nor a mixture of the adult fast and slow types. Whalen et al. (137) suggested that the conflicting results of the immunocytochemical studies may be due to differing degrees of cross-reactivity of the antibodies with myosin analogous to the embryonic and neonatal forms. Whether the results obtained in rat skeletal muscles apply to muscles of other species, remain to be determined.

Myosin is composed of two heavy chains (200,000 molecular weight) and another four polypeptides varying in molecular weight between 15,000 and 27,000, called the light chains (LC). In general two LC of fast muscle type, LC1f and LC2f (114, 138) and one embryonic LC (LC1emb) are characteristic of the early stages of rat muscle development (135). Small quantities of a LC of slow muscle type (LC1s) have been observed (26, 65, 89, 123). Obinata et al. (89) demonstrated that during early stages of development of chick embryos the breast and leg muscles contain LC1', in addition
Myogenesis in Vitro

99

to fast-type myosin LC, but as development proceeds, the fast-type LC predominate in these muscles. In primary muscle cultures of calf, the LC2t is the first light chain synthesized 1 day after fusion; then at day 3 the LC1't, LC1't, and the LC1_emb are detected. Whereas the synthesis of most myosin light-chains remains approximately constant after day 5, the embryonic type of LC (LC1_emb) decreases after this stage. Messenger RNAs coding for LC1't, LC1't, and LC1_emb are not detectable by in vitro translation of cytoplasmic RNA extracts prepared from cells 1 day after fusion. Whereas mRNA for LC2t appear to be present in these cells (26).

Summarizing, it seems that the main increase in protein synthesis takes place at about the time of cell fusion, and that the corresponding translatable mRNA also accumulates during this stage (26, 27).

b) ACh receptors. Another characteristic of mature muscle is sensitivity to ACh. Since this ACh-dependent membrane depolarization is mediated by specific membrane proteins, one may monitor the appearance of these receptors as an indicator of muscle differentiation.

There are two general methods to assay the presence of the receptors. In the first, one intracellularly records the time course of the muscle membrane potential during iontophoretic ACh application (32, 34). In the second method, one applies [125I]-alfa-bungarotoxin, and the extent of toxin binding is determined by scintillation counting of scraped cultures (96), or by autoradiography of fixed cultures (49). The snake toxin can also be conjugated to rhodamine or fluorescein, and the receptors visualized in a fluorescence microscope (1,120). ACh receptors appear early during myogenesis. They have been identified in mononucleated myoblasts and the receptor density increases to about 1,000 binding sites/µm² as the cells fuse to form multinucleated myotubes. There is evidence that receptor incorporation is associated with fusion. Counts made of toxin binding in chick and rat muscle cultures indicate that the amount of toxin bound per plate rises 8- to 10-fold during myoblast fusion. This raises the question of whether receptor formation (or activation) is dependent upon fusion. Replacement of normal medium with low calcium (25 µM) medium one day after plating decreases the fraction of fused nuclei to 15%, in eight hours, compared to 65% in controls. In chick myotubes the total amount of toxin bound per plate rises at a similar rate in both cases indicating that receptor production is not dependent upon fusion (79, 96, 103). However, a strong dependence upon fusion has been observed by Shainberg and Brick (115) in rat myotubes. We have recently found that in C5, a mouse muscle cell line, low calcium medium decreases total receptors by 20–50%, and disperses ACh receptors clusters (57), an effect also observed in rat embryonic muscle (9, 10).

Clusters of ACh receptors detected by toxin binding or ACh sensitivity have been found in primary cultures of chick and rat muscles (36) and in a myogenic cell line (57). Because local areas of high ACh receptors density are characteristic of adult neuromuscular junctions, it is tempting to suggest that ‘clusters’ on myotubes may function as targets for invading nerves and hence form the postsynaptic precursors of the motor endplate (35, 128). Recent work, however, has demonstrated that although ‘clusters’ are present before innervation, new areas of high sensitivity develop at nerve-muscle contacts (1, 41). It should be noted that myotubes developing in vivo do not accumulate several patches of receptors as happens in culture. In contrast, the region of the neuromuscular junction is the only one with increased receptor density (6, 16). However, denervated adult fibers do develop additional receptor
clusters (69), and multiple ACh receptor clusters also appear on inactive rat embryonic myotubes (152). Despite the fact that multiple `clusters' are not normally found on innervated developing muscle in vivo, their appearance in cultured fibers provides a convenient system for the study of mechanisms involved in the stabilization of groups of receptors.

The incorporation of new ACh receptors into membranes of cultured cells and their subsequent metabolism has been studied with receptors labelled with radioactive alfa-bungarotoxin, radioactive aminoacids, and heavy isotopes. Receptors are synthesized by an energy-dependent process that can be inhibited by blockade of protein synthesis or by inhibition of RNA synthesis (49). After treatment with such an inhibitor it takes about 2 hours before the appearance of new surface receptors is blocked, indicating that there is an internal pool of newly synthesized receptors (29). Merlie and Sebbane (81) have shown that newly synthesized ACh receptor subunits require approximately 15–30 min before acquiring alfa-bungarotoxin binding activity, thus indicating the existence of a pool of inactive precursors at the stage that precedes the pool defined by Devreotes et al. (29). Merlie et al. (82), using immunoprecipitation and SDS gel analysis, have also shown that the membrane-bound poly-ribosomes direct the synthesis of the alfa-subunit of ACh receptor. Furthermore, peptide maps suggest that two protein species (39,000 and 42,000 molecular weight) synthesized in vitro may correspond to the non-glycosylated and glycosylated forms of the alfa-subunit. The post-translational modification apparently occurs before/or in the Golgi apparatus, because 125I-alfa-bungarotoxin binding sites have been localized by autoradiography in this organelle (33). New ACh receptor are inserted into the membrane at a rate of 90 receptors/μm²/h. Degradation of receptors has been found to be an energy-dependent proteolytic process, which involves movement of the ACh receptors inside of the cell (28). The half-life of ACh receptors on chick and rat myotubes has been estimated to be 15–20 hours (31). On the other hand, antibodies obtained from sera of patients with myasthenia gravis increase the ACh receptor degradation rate both in vivo (110) and in vitro (3).

VI. Differentiation of skeletal muscle from non-muscle precursors

The mesodermal origin of skeletal muscle is universally accepted. Several lines of evidence, however, suggest that neuroectoderm may also have the potential to give rise to skeletal muscle. For example, striated muscle cells have been observed in the human central nervous system, in association with congenital anomalies (52). Muscle elements have been found as a rare event in normal mammalian pineal (50), cerebelum (90), leptomeninges (74) and cerebrum (132).

Lennon et al. (73) have observed the spontaneous change of two rat glial-like clonal cell lines into muscle cells. These cells fused into contractile multinucleate fibers and incorporated ACh receptors on their plasma membrane. Several groups of investigators have reported the occurrence of striated muscle fibers in primary cultures of pineal glands (42, 87, 111). Pineal myotubes were shown to contain the thick and thin filaments characteristic of striated muscle (111), and were electrophysiologically and pharmacologically similar to skeletal muscle fibers kept in vitro (42). The resting membrane potential increased with age in the culture, a normal feature of chick and mouse cultured myotubes (37, 101). All pineal myotubes showed delayed rectification, and action potentials occurred either spontaneously or could be evoked if the mem-
brane was sufficiently hyperpolarized. The myotubes also possessed nicotinic ACh receptors, and ionophoresis of ACh showed heterogeneous sensitivity, suggesting clustering of ACh receptors (42). Recently Wier and Lennon (139) have shown that regular development of contractile muscle fibers arises from primary cultures of rat optic nerve. It was shown that cells dissociated from optic nerve were sparse for the first 6 days in culture, but by day 10 were confluent, and multinucleated fibers began to appear. Fibers increased in size in the subsequent weeks and many became spontaneously contractile. Striations were readily observed with phase contrast microscopy. Together with the morphological differentiation these cultures developed ACh receptors, and the total number of toxin-bound to the cells increased more than 30-fold during muscle differentiation.

All of the above findings clearly suggest that mammalian cephalic neuroectoderm has myogenic potential. The possibility that muscles localized in separate anatomic sites could have different embryonic origins might explain the preferential involvement of discrete muscles in certain pathologic conditions.

VII. Tissue culture studies in normal and abnormal human skeletal muscles

In the past several years many laboratories have examined tissues from several organ systems aiming to understand the inborn errors of metabolism in different kinds of muscular dystrophies. Although results have often been equivocal, most recent studies have failed to show any morphological differences between normal and abnormal muscles. Almost all these studies have involved techniques based on explants of small muscle pieces in the presence or absence of nerve (5, 140, 141). The failure to demonstrate differences in explants of dystrophic muscle may result from preselection of cells in the early stages in vitro. For example, dystrophic cells may fail to leave the explant and only relatively healthy cells ultimately may migrate out and contribute to myotube formation. Alternatively, the cells that ultimately do form myotubes may not be capable of differentiating in vitro into mature fibers. If the defect is only expressed in the mature skeletal myotube, tissue culture would hardly reveal the abnormality. A new cell technique, which involves tryptic digestion and mechanical dissociation (148, 149), has been used successfully in the growth of muscle cells from a mononuclear cell suspension prepared from adult human skeletal muscle. This technique allows quantification of the number of mononuclear cells obtained per gram of tissue, as well as of plating efficiency (149).

Using this technique, Thompson et al. (130) reported that Duchenne dystrophic cells differ from normal ones. During the proliferation and migration of mononuclear cells, Duchenne cultures showed impaired contact inhibition and the presence of many small clumps of cells. As myoblast fusion proceeded, the developing myotubes proliferated between the clustered cells. At an ultrastructural level the Duchenne mononuclear cells possessed abnormal vacuoles (129, 150). Tissue cultures from myotonic muscular dystrophy and Duchenne muscular dystrophy have been compared to normal myotubes with respect to electrophysiological properties using the technique of Yasin et al. (149). No abnormalities have been observed in the Duchenne muscular dystrophy myotubes (4). However, in myotonic muscular dystrophy myotubes several changes have been noted. The most distinctive abnormalities are: an increased tendency to fire repetitive action potentials, decreased resting membrane potential, decreased action potential amplitude and overshoot, decreased action po-
tential after-hyperpolarization, and decreased outward-going rectification determined from steady-state current voltage plots. Although the specific molecular explanation for these electrical changes is not available, an abnormality in outward-going potassium current—especially that turned on by calcium—provides a reasonable hypothesis to account for these abnormalities in myotonic muscular dystrophy (77, 78).

The primary importance of the above studies is to support the value of muscle culture in the morphological and electrophysiological analyses of the muscular dystrophies. These cultures should also prove of considerable value in establishing the biochemical consequences and the primary inborn errors of metabolism that result from genetic defects.

VIII. Conclusion

The use of muscle cells in culture should facilitate studies on the development of complex phenomena such as electrical excitability and excitation-contraction coupling and should also make possible to investigate the effects of innervation using combined nerve-muscle cultures.

Tissue culture, as with any technique, has its advantages and limitations. Its most appealing aspect is that of dealing with living functional cells in a defined environment outside the body. However, this is a double-edged sword. In removing the cells from a complex environment of changeable cell populations and from variable volumes of extracellular fluids, one may have also removed the specific cues that instruct cells in their particular duties of producing a final differentiated function (129). In the specific case of muscle cells, they may have been removed from the signals of neighboring nerve cells that could have instructed an alternative behavior. The problem is to convince the cells that they are still in vivo by supplying them with appropriate cues, requirements, or any other differentiation factors that will induce them to behave in vitro with their muscle-specific behavior, i.e., synthesizing muscle-specific proteins (ACh receptors), and organizing them into appropriate form (clustering of receptors). Recent studies of muscle cells in culture indicate that spinal neurones (61, 100) and neuroblastoma-glioma hybrid cells (19), release a factor that induces clustering of receptors in adjacent myotube membranes.

Various investigators have shown that muscles and nerves do form synapses in vitro, in which transmitter’s release from the nerve leads to excitation and contraction of the muscle (38, 48, 85, 88). Formation of junctions in vitro tends to be inefficient and highly asynchronous (41, 88), similar to immature junctions in the developing animal (85). Nonetheless, they have demonstrated that the in vitro approach to neuromuscular junction formation is likely to be fruitful.

The early literature on the formation of neuromuscular junctions, both in cell and organ culture, has been reviewed by Shimada and Fischman (119). Recent studies on mixed nerve and muscle cultures have revealed that:

1. Muscle impulse and contractile activity is unnecessary for the development of synaptic ultrastructure or for the localization of ACh receptors (2, 21).
2. The localization of ACh receptors at nerve-muscle contacts is nerve-induced and involves a redistribution of surface receptors (1, 23, 41).
3. The development of synaptic specializations does not occur at nerve-muscle contacts when the source of nerve is dorsal root ganglion or sympathetic ganglia, rather than spinal cord (22, 24).
(4) The ability of a nerve to cause accumulation of ACh receptor is separate from that to form the functional synapse (66).

There are hazards involved in tissue culture that should be taken into consideration. Disaggregation of muscle by use of trypsin or other proteolytic enzymes may have deleterious effects on the properties of the membrane, whose variable recovery may bias the results of a developmental study. A clear example has been observed in the case of ACh receptors, which are lost in chick embryo myoblasts prepared by trypsinization (121). As I discuss in the beginning of this summary review, one of the traditional methods for selecting myoblasts as opposed to fibroblasts is the pre-plating technique, which is based on the differential adhesion of fibroblasts to the plastic substrate. There is, one caveat concerning the presumed selection of myogenic cells, because in the case of myoglobin it has been shown that preplating does not achieve an enrichment but instead a loss of the 50% of the myoglobin producing cells (62).

Acknowledgments. I thank Dr. Fabian Jaksic, University of California, Berkeley, for his comments on the manuscript. Support during preparation of this review was provided by a Muscular Dystrophy Association Postdoctoral Fellowship in the Department of Physiology, University of California, San Francisco. Additional support was obtained through grants from the National Institutes of Health, the Muscular Dystrophy Association, and the National Science Foundation to Dr. Zach W. Hall.

REFERENCES


Myogenesis in Vitro

42. FRESCHI, J.E., A.G. PARFITT and W.G. SHAIN. Electrophyiology and pharmacology of striated muscle fibres cultured from dissociated neonatal rat pineal glands. J. Physiol. (Lond.) 293, 1–10, 1979
44. HAUSCHKA, S.D. Clonal analysis of vertebrate myogenesis. II. Environmental influences upon human muscle differentiation. Develop. Biol. 37, 329–344, 1974
47. HARRIS, A.J. Embryonic Growth and Innervation of Rat Skeletal Muscles. II. Neural Regulation of Muscle Cholinesterase. Phil. Trans. R. Soc. (Lond.) B, 293, 279–286, 1981
60. ISHIKAWA, H. Formation of elaborate networks of T-system tubules in cultured skeletal muscle


76. MASAKI, T. and C. YOSHIZAKI. Immunochemical comparison of myosins from chicken cardiac, fast white, slow red, and smooth muscle. J. Biochem. 76, 123–131, 1974


81. MERLIE, J.P. and R. SEBBANE. ACh receptors subunits transit a precursor pool before acquiring alfa-bungarotoxin binding activity. J. Biol. Chem. 256, 3605–3608, 1981a


86. NAMEROFF, M. and E. MUNAR. Inhibition of cellular differentiation by phospholipase C. II. Separation of fusion and recognition among myogenic cells. Develop. Biol. 49, 288–293, 1976
89. OGINATA, T. T. MASAKI and H. TAKANO. Types of myosin light chains present during the development of fast skeletal muscle in chick embryo. J. Biochem. 87, 81–88, 1980

110. REINESS, C.G., C.B. WEINBERG and Z.W. HALL. Antibody to acetylcholine receptor increases degradation of junctional and extrajunctional acetylcholine receptors in adult muscle. Nature (Lond.) 274, 68–70, 1978


120. STOCKDALE, F.E. Changing levels of DNA polymerase activity during development of skeletal muscle in vivo. Develop. Biol. 21, 462–474, 1968


123. SUGIYAMA, H. Multiple forms of acetylcholinesterase in clonal muscle cells. FEBS Lett. 84, 257–260, 1977


131. VOGEL, Z., A.J. SYTKOWSKI and M.W. NIRENBERG. Acetylcholine receptors of muscle grown


147. YAFFE, D. and M. FELDMAN. The formation of hybrid multinucleated muscle fibers from myoblasts of different genetic origin. Develop. Biol. 11, 300–317, 1965


(Received for publication, April 5, 1982)