Development of Vertebrate Skeletal Muscle

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SYNOPSIS. An investigation of developing skeletal muscle necessitates the study of three categories; the derivation of muscle cells or fibers, myofilament synthesis and interactions, assembly of myofilaments into functional sarcomeres of striated myofibrils. With few exceptions, skeletal muscle cells are of mesodermal origin, and consist of rounded mononucleated cells which elongate and fuse with one another to become myotubes. Within the sarcoplasm, myofibrillar proteins are synthesized and grouped into interacting thick and thin filaments. Crude, non-striated myofibrils result from linear arrangements of thick and thin filaments which are horizontally aligned by the invaginating sarcotubular system. After Z-lines form, providing attachment sites for thin filaments, a typical banding pattern follows. The newly formed Z-lines pull apart, followed by the attached thin filaments, and repeating "relaxed" sarcomeres are the resulting striated myofibrillar pattern.

INTRODUCTION

Skeletal muscle is composed of specialized, multinucleated, cylindrical cells or fibers which display alternating light and dark bands. The striated or banded appearance is created by repeating subunits called sarcomeres, demonstrated just thirty-five years ago to be composed of small filaments, or myofilaments (Hall et al., 1946).

When discussing the development of skeletal muscle, one must give major concern to three categories: (1) the muscle cell—where it is embryologically derived and how are its developmental stages characterized, (2) the myofilaments—how are they synthesized and when do they appear during development, and (3) the myofibril—how do the myofilaments fit together to form a functional sarcomere. Excellent literature reviews concerning different aspects of these three questions are readily available (Boyd, 1960; Holtzer, 1961; Fischman, 1972; Allen, 1973a). In this symposium, I will discuss briefly some aspects of these investigations as well as others, and attempt to put some of the conflicting studies together into a concise story.

Before beginning the discussion of developing skeletal muscle, a brief review of the structure and composition of mature myofibrils is necessary. In a light micrograph of muscle (Fig. 1), longitudinal striations are visible due to the parallel arrangement of myofibrils. Each fibril is composed of periodic sarcomeres which are in turn composed of myofilaments. These sarcomeres, in lateral register with one another, create the transverse striations or banding patterns which are clearly seen in electron micrographs (Fig. 2) of skeletal muscle. The distance from one dense Z-line to another includes one sarcomere. The darker band found in the center of the sarcomere is the A band, with less dense I bands on either side. The H zone is the light area in the center of the A band, bissected by a dense M-line. The longitudinally oriented sarcoplasmic reticulum and its relationship to the transverse T-tubules is clearly indicated. Elongate mitochondria are also apparent in this fully differentiated skeletal muscle from the nine-banded armadillo (Ruby and Allen, 1976). Two distinct classes of myofilaments (thick and thin) have been isolated and characterized both from adult skeletal muscle and from purified preparations of others.
myosin and actin (Huxley, 1961; Hanson and Lowy, 1963). The A band is composed of thick myosin filaments approximately 1.5 \mu m long and 11-12 nm in width (Figs. 2,4). Thick filaments are continuous from one end of the A band to the other. The M-line is apparently purely structural in nature and functions to help preserve the integrity of the thick filaments (Kundrat and Pepe, 1971). Thin filaments approximately 6 nm in width consist of two helically wound strands of 5 nm actin monomer subunits. Filament lengths measure approximately 1 \mu m (Pepe and Huxley, 1964). Unlike the thick filaments, which are thought to contain myosin molecules, the thin, actin filaments are known to have other minor but necessary contractile proteins associated with them. Tropomyosin is in the form of a strand, lying in the groove created by the tightly bound actin filaments (Ebashi and Endo, 1968). Troponin is a complex structure distributed at 38.5-4.0 nm intervals along the thin filament (Ebashi and Endo, 1968). The Z-line contains a dense proteinaceous material that affords attachment of thin filaments, either by “Z” filaments” (Knapppeis and Carlsen, 1962) or by an intertwining mechanism (Kelly, 1969). α actinin is
SKELETAL MUSCLE DEVELOPMENT

With few exceptions (See Boyd, 1960) skeletal muscle is of mesodermal origin. Narayanan and Narayanan (unpublished observations), however, recently described a striated muscle of the iris in birds that is innervated by visceral efferent nerves and derived from neural ectoderm. In contrast to smooth muscle fibers found in the mammalian iris, striated fibers are essential in birds because of the necessity of quick light adaptability during flight. Other skeletal muscle found in the head includes two embryologically different groups. One group is from visceral (branchial) arch mesoderm and gives rise to masticatory, laryngeal, pharyngeal, and mimetic muscles. The other group is from myotomes (found within somites) and gives rise to the extraocular and tongue musculature. According to Huber, (1931), certain muscles of the shoulder also belong to this latter group. The remainder of the trunk musculature is derived from somites and lateral plate mesoderm (Strauss and Rawles, 1953) or exclusively somites (Detwiler, 1955; Theiler, 1957; Liedke, 1958). Limb musculature is derived from local condensations of lateral plate (somatopleuric) mesoderm. (See review by Fischman, 1972).

Undifferentiated mesenchymal cells, destined to become muscle, are not easily identified from the other embryonic mesenchymal cells. In general they are of a round shape containing an ovoid nucleus with one or two prominent nucleoli. They also contain numerous free ribosomes and short polyribosomes, and usually a small amount of poorly developed rough surfaced endoplasmic reticulum. The cells elongate and become bipolar, in contrast to triangular or polygonal shaped mesenchymal cells that will not become muscle. Capers (1960) and Cooper and Konigsberg (1961) observed mononucleated muscle cells fusing to become multinucleated myotubes in culture, recording the act in the time lapse cinematography. Muscle cells are readily identified in the fusion process in vivo when we are utilizing 3-4 day chick embryos (Stage 20) in our laboratory. The method by which myotubes become multinucleated has been proposed by Holtzer et al., (1957) to be by the fusion of mononucleated myoblasts with other myoblasts or myotubes and not by mitotic, amitotic or some other means (see Fischman, 1972). A confusing terminology for the developing muscle cell has appeared in the literature since Godlewski first introduced the term "myoblast" in 1902 (see Boyd, 1960). Holtzer and colleagues (see Holtzer, 1970) proposed a widely accepted nomenclature. They term any postmitotic, mononucleated cell, capable of both fusion and the synthesis of contractile proteins a "myoblast." They further state that the myoblasts are immediate descendants of "presumptive myoblasts" which cannot fuse nor synthesize myosin.

Although some evidence has been presented for myofibrillar protein synthesis in mononucleated cells (Holtzer et al., 1957; Przybylski and Blumberg, 1966; Fambrough and Rash, 1972), it appears that myotubes are the location of the bulk of myofibrillar protein synthesis and assembly of myofilaments (Fischman, 1972). With the initial onset of myosin synthesis, large polyribosomes containing 50-60 ribosomes in a chain and shown to be actively synthesizing myosin (Allen and Terrence, 1968) are found in the sarcoplasm of myotubes. There is also an increase in length and number of mitochondria observed in the myotube, both in electron microscopy of in vivo studies (Allen and Pepe, 1965) and from enzyme histochemical data of in vitro investigations (Cooper and Konigsberg, 1961). Long polyribosomes, elongated mitochondria and early stages of myofibrillogenesis are seen in Figure 3. Suggestions of cell fusion are also indicated in these embryonic chick cells. The surface of the myotube is coated at an early developmental stage with a glycocalyx called a basal lamina. Okazaki and Holtzer (1966) and Fischman (1970)
FIG. 3. Low power electron micrograph of a differentiating muscle cell containing a nucleus (N), elongated mitochondria (m), long polyribosomes (arrows) and an early nonstriated myofibril. Areas of probable fusion are indicated by the large arrowheads. × 6,000. (After Allen and Pepe, 1965).

noted that myotubes in vitro possess a basal lamina after the fifth day in culture. Mononucleated cells are devoid of this surface coat, hence its presence is suspected of preventing further developmental fusion (Fischman, 1972). An ultrastructural feature of the cultured myotube is the numerous free 5-6 nm cytofilaments or cortical filaments found not to bind HMM antibody as does actin (Ishikawa, et al., 1969). Since these filaments are found particularly near the ends of growing myotubes and also in nonmyogenic cells they are thought not to be involved in myofibrillogenesis.

SYNTHESIS AND APPEARANCE OF MYOFILAMENTS

Early investigators of muscle differentiation were extremely limited by the techniques available to them. The resolving power of the electron microscope, necessary to see myofilaments within myofibrils, did not enter the field until the middle 1940's (Hall, et al., 1946).

Prior to investigations of rat skeletal muscle morphogenesis by Bergman (1962), myofibrils were believed to be initially formed by some mysterious sarcoplasmic condensation and polymerization (Van Breeman, 1952; Hibbs, 1956; Goldman, 1958). Waddington and Perry (1963) reported the presence of long helical arrangements of ribosomes in the sarcoplasm of developing frog skeletal muscle cells. Behnke (1963) simultaneously reported these ribosomal configurations in differentiating small intestine cells of rat fetuses. Later that same year, working with rabbit reticulocytes, Rich coined the term polyribosomes for chains of protein synthesizing ribosomes (Rich 1963). Heusson-Steinnon (1964), described a close association between polyribosomes and developing muscle protein. In 1965, Allen and Pepe showed a morphological correlation between the appearance of thick, myosin myofilaments and long polyribosomes containing 50-60 ribosomes in a chain. Heywood and co-workers (1967) isolated and biochemically identified nascent myosin from the long polyribosomes. Immunological methods were also used to identify the nascent myosin (Allen and Terrence, 1968), using an antibody previously prepared against and shown to be selective for localizing myosin within embryonic chick somites (Holtzer, et al., 1957). The studies of Heywood and Rich (1968) demonstrated the synthesis of myosin, actin, and tropomyosin to be on polyribosomes of sizes expected from the molecular weights of the respective subunits.

Myosin

Nascent peptides of varying size ranges interact to form myosin molecules. Obinata et al., (1966) reported two sizes of myosin (light and heavy) in embryonic chick muscle and suggested the smaller of the two gives rise to the larger. The size of a peptide synthesized by a polyribosome of
the size indicated by Heywood et al. (1967) and Allen and Terrence (1968) has been correlated with the heavy subunit of myosin (Fredericksen and Holtzer, 1968; Lowey, et al., 1969; Paterson and Strohman, 1970). When muscle cultures were used as a model, one set of myosin subunits were found in mononucleated cells and yet another size range in multinucleated cells. Others were common to both systems (Chi et al., 1975). Although much useful information is obtained in vitro, the studies of Hilfer et al. (1973) and Holtzer et al. (1975) have emphasized the caution that should be taken when attempting to extrapolate in vitro experimental results with normal events occurring in vivo. Disregarding the correlation of initially appearing myosin subunits in vivo with the tryptic digestive products light and heavy meromyosins, we know the total myosin molecule is a very large protein possessing a molecular weight of approximately 470,000. We can also calculate the number of molecules in a thick filament to be around 400. Since thick filament fragments are not observed in vivo, there must be a very rapid assembly of the myosin molecules within the thick filaments. Figure 4 represents an electron micrograph from a negatively stained homogenate of an embryonic chick somite. A thick and part of a thin filament are seen side by side.

**Actin**

In 1949, Kesztysus, et al. prepared antibodies to the two major muscle proteins. Localization studies done by tagging the antibody with fluorescein isothiocyanate, placed actin with I bands (Holtzer, et al. 1957). There has been much controversy over the time of the initial appearance of the protein and or the 6 nm filaments containing actin ever since. The early studies of Holtzer et al. (1957) demonstrated a simultaneous appearance of myosin and actin filaments during myogenesis in cultured muscle although later studies of developing muscle in vitro indicated an earlier appearance of actin (Heywood and Rich, 1968). Ultrastructural studies did not clarify the contradictions, as studies of salamander muscle (Hay, 1963) and chicken leg muscle (Fischman, 1967), both indicated a simultaneous appearance of the two muscle filament sizes (thick-myosin, thin-actin). Other studies, however, have indicated an earlier appearance of the thin, 6 nm, actin filaments (Allen and Pepe, 1965; Obinata et al., 1966; Przybylski and Blumberg, 1966; Hilfer et al., 1973; Murphy, 1977). Biochemical studies, using precipitin tests, had previously shown actin to appear prior to myosin in developing whole chick embryos (Ogawa, 1962), when testing for free molecules as well as myofilaments.

Numerous studies have shown actin (Tilney, 1976-1977), as well as myosin, (see review by Pollard and Weihog, 1974) to be a protein constituent of various non-muscle cells. In culture, these cytofilaments are found predominantly beneath plasma membranes, primarily at the ends of growing myotubes. They are suspected to be involved in cytoplasmic streaming (Fischman, 1972). Ishikawa (1968) and Kelly (1969) further complicated the myofilament controversy by describing 10 nm filaments called “intermediate filaments” that do not bind the antibody to heavy meromyosin as does actin (Ishikawa et al., 1969). The intermediate size filaments have also been observed in non-muscle cells (Ishikawa et al., 1969), but have not been encountered in more recent in vivo studies (Hilfer et al., 1973; Murphy, 1977). This author believes, as suggested earlier (Allen, 1973a; Hilfer et al., 1973), that slight differences found in in vivo and in vitro myogenic processes, which ultimately lead to identical sarcomeres, may account for varying sequential differences described in the literature. Observing negatively stained homogenates as well as routinely embedded and sectioned chick somites, Allen and Pepe (1965) demonstrated an early myogenic age where thin but no thick filaments could be seen. The earlier appearance of actin filaments in developing chick myotome cells is observed routinely in my laboratory and many of these randomly oriented thin filaments virtually saturate the sarcoplasm of in vivo somitic muscle cells prior to the
FIG. 4. Electron micrograph of a negatively stained homogenate from somites of a 3 day chick embryo. Approximately 400 myosin molecules are present in the isolated thick filament. The broken thin filament contains 2 helically wound strands of globular actin monomers, a strand of tropomyosin, and periodic troponin molecules. $\times 140,000$ (After Allen, 1973a).
appearance of thick filaments. The 6 nm thin filaments are morphologically identical to those isolated from adult skeletal muscle and purified actin preparation (Hanson and Lowey, 1963; Allen and Pepe, 1965).

**Minor muscle proteins**

Tropomyosin and troponin are regulatory proteins closely associated with the thin, actin filament. Very little has been reported concerning their development. Heywood and Rich (1968) found polyribosomes, presumably synthesizing tropomyosin in vitro, appearing in development during later stages of embryogenesis than either actin or myosin. In cervical somites of chick embryos, Hirabayshi (1971), detected the presence of tropomyosin at the same time as actin and myosin. Results by Hitchcock (1970), indicate a deficiency in both tropomyosin and troponin prior to 14 days of incubation, 11-12 days after actin and myosin have been detected. Fischman (1972), suggested the possibility of the two regulatory proteins being present but in an inactive form. This suggestion appears very probable since a definite correlation exists between troponin, which is associated with tropomyosin, and the sarcotubular system (Hitchcock, 1970). The tubules are not organized into a final form reported for adult skeletal muscle, until late in development. Recent studies of chick dystrophic muscle support this possibility (Murphy, 1977). Murphy (1977) found a lack of an organized sarcotubular system and scarcity of troponin antibody binding in developing dystrophic chick embryos as compared with similar age normal chick embryos.

Other minor muscle proteins have been isolated from myofibrils, viz, α actinin, β-actinin, M-protein, but studies concerning their development have been neglected. It has been demonstrated, however, that M-lines, containing M protein, are detected within sarcomeres of the chick just prior to or almost simultaneous with the appearance of typical cross striations (Allen and Grisnik, 1971).

**The myofibril**

Thin, actin containing filaments approximately 6 nm in diameter, appear in developing chick skeletal muscle prior to myofilaments of any other size range. The actin filaments increase in number and virtually saturate the sarcoplasm of myogenic myoblasts before a single thick (myosin) filament can be seen. When only a few initial thick filaments are detected, they are not observed singly, but surrounded by many thin ones (Allen and Pepe, 1965; Fischman, 1967). Fischman (1967) suggests that myofilament properties leading to aggregation may also be responsible for spatial arrangements. In an investigation of actin filament polarity during initial stages of myofibril assembly, Shimada and Obinata (1977) found thin filaments exhibiting the right polarity and spatial position similar to that seen in mature muscle. They suggested an immediate interaction between thick and thin filaments similar to that indicated earlier by Allen (1973b). This initial attraction (actomyosin reaction) would inevitably lead to an early hexagonal array of thin filaments surrounding thick ones.

Soon after a cluster of hexagonally arranged thick and thin filaments appear, they become linearly arranged in the longitudinal axis of the cell, predominantly near the cell's periphery (Allen and Pepe, 1965). The mechanism of linear grouping (see Fig. 3) is not known. Allen and Pepe (1965) showed the crude myofibrils to be traversed by membranous tubules at regular periodic intervals of 1.5 μm, forming segments comparable in length to a thick filament and/or an A band. This description of non-striated myofibrils forming prior to striated ones is not a new concept (Godlewski, 1902; Weed, 1936; Holtzer et al., 1957; Allen and Pepe, 1965; Allen, 1973b). Holtzer (1961), stated in a review of myogenesis, that every striated myofibril is preceded by a brief non-striated period. Ezerman and Ishikawa (1967), first demonstrated a tubular system, thought to be the T-tubule component of skeletal muscle, formed by inward invaginations and branchings of the sarcolemma in develop-
ing muscle cultures. These studies were the first of many such investigations (see review by Franzini-Armstrong, 1972). Early nonstriated myofibrils are often found near the periphery of myogenic cells, thus the invaginating tubules (Ezerman and Ishikawa, 1967; Allen 1973b), may be instrumental in the initial myofibrillar alignment. Allen and Pepe (1965), using chick muscle and Walker and Edge (1971), using rat muscle, demonstrated that the periodic tubules of the early myofibril are followed by a close association of a Z-line material. Studies of guinea pig, rabbit, cat, dog, goat, and sheep cardiac muscle (Sommer and Johnson, 1968) as well as amphibians (Warren, 1973), have all indicated a close association between an enveloping tubular system and early Z-lines. This association is indicated in Figure 5. Many workers, both in light and electron microscopy, have demonstrated that a Z-line is the first form of a cross striation to appear on myofibrils (Weed, 1936; Van Breeeman, 1952; Hibbs, 1956; Shafig, 1963). The inpocketing, branching and enveloping tubular system may also explain the lateral register seen in Z lines on neighboring myofibrils (Walker et al., 1968). In the studies of Walker and Edge (1971), it was further shown that electron-opaque strands were seen connecting the tubules to forming Z-lines. The strands may represent the thin "Z filaments" (Knappeis and Carlsen, 1962), or the connecting mechanism proposed by Kelly (1969), for connecting thin filament tips to Z bands. If there is an attraction as well as connection function, then these filaments may help to explain the observed linear aggregating of hexagonally arranged myofilament clusters. The longitudinal growth by merestimatically adding new non-striated sarcomeres to the ends of striated fibrils (Holtzer, 1961), adds strength to this possibility. This author feels, as first suggested in 1965 (Allen and Pepe, 1965) and again in 1973 (Allen, 1973b), that these early Z bands form an attachment or anchoring point for the thin, actin myofilaments. The passive pulling apart of 1.5\(\mu\)m periodic Z lines (thick filament length) during growth would give a typical A, I, H banding pattern. As demonstrated in Figure 6, the I bands would form on either side of the central A band and an H zone would be visualized in the center. Both bands are and would be the result of thin filaments sliding past thick ones to follow attached Z lines. Holtzer (1970) stated that relationships between thick and thin filaments necessary to form a central H band in the sarcomere, may be dependent upon the presence of the Z line. Using antibody staining, he also could detect developing A bands prior to visualizing Z lines with

FIG. 5. Electron micrograph of an early myofibril striated only with a periodic tubular system and associated Z-line material. Note the close association between the two. x 36,000.
FIG. 6. Diagrammatical representation of sarcomere formation in vivo. (a) Initial appearance of 1.5 μm segments (A band length) traversed by a periodic tubular system. (b) Z-lines have formed in association with the tubular system and provided anchor points for thin filaments. Z lines pull away and result in a "typical" striated banding pattern.

Phase microscopy. A pulling apart of the Z-lines and thus the filaments would increase the size of the sarcomere, and this is the recorded observation (Allen, 1973a). A developmental sequence such as the one described previously would mean that our terminology for aspects of muscular contraction may develop backwards. Skeletal muscle sarcomeres first appear in the "contracted" state, and secondarily, due to Z lines pulling apart, become "relaxed."

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