Studies on Development and Differentiation of Muscle III. Especially on the Mode of Increase in the Number of Muscle Cells

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Tateki KIKUCHI

Department of Animal Science, Faculty of Agriculture, Tohoku University, Sendai, Japan

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Summary

The mode of increase in the number of skeletal muscle cells (*M. complexus and M. biceps femoris*) in chick embryos was studied with the electron microscope and light microscope.

For the electron microscopic analysis, the *M. complexus* samples were prepared at 12, 14, and 16 days of incubation. Various cell types in the muscle tissue were described at various stages of development. Myotubes, consisting of the primary myotube and a number of successive generation myotubes in one muscle cell cluster, have special plasma membrane attachments. They display invagination and diffuse, but not continuous between neighboring muscle cells. This phenomenon was encountered only in limited areas and never extend over the full length of the myotube.

It may be concluded that myoblasts proliferating along the wall of the myotube do not fuse laterally to other differentiated myotubes, but play important role in the separation of each neighboring myotube by way of extending a pseudopoidal process.

The mode of myogenesis in the *M. complexus* which enlarge in diameter of muscle cells at the later developmental stages was compared with the *M. biceps femoris* by the use of a light microscope. The primary myotubes in the *M. complexus* show a slight enlargement in diameter, but are nearly equal between both muscle tissues during all stages of development. The successive generation myotubes in the *M. complexus* differentiate and enlarge more rapidly after 12 days of incubation than those in the *M. biceps femoris*.

On the basis of these results, it seemed to be reasonable to conclude that the successive generation myotubes arise from the fusion process of myoblasts proliferating along the wall of the primary myotube, and that they separate from the surface of the primary myotube.

The mode of increase in the number of skeletal muscle cells in the fetal stage has been studied in relation to their multinuclearity. It was originally reported that multinculeated muscle fibers arise from individual cells proliferated by amitotic division (1–6). This *Unicellular theory* was accepted from the ninteenth to the

middle of this century by many investigators who studied the amitosis process of the regeneration of muscle tissue (7, 8).

The other *Multicellular theory* claimed that multinucleated muscle fibers spring from mononuclear cells by the coalescence process (9, 10). This theory has been neglected for a long time. Lash et al. (11) reported that nucleic divisions were found only in mononuclear cells, but not in multinuclear myotubes and that no sign of amitotic division was ever visible in the cytophotometric analysis studies on mouse regenerating muscle. The *Multicellular theory* seems to have been settled by the works of recent investigators using the muscle tissue culture method (12–20).

On the other hand, the problems of increase in the number of muscle cells have been studied by many investigators who were opposed both points of view. Firstly, some authors thought that the new muscle cells arise by the splitting of or by budding from already differentiated striated muscle cells. Some others, however, have supported those who think that new cells are added to the developing muscle from some of the satellite cells until the full number is achieved.

In the following paper, it is my purpose to trace morphologically the mode of increase in the number of muscle cells by means of electron microscope and light microscope.

Materials and Methods

Skeletal muscle (*M. complexus* and *M. biceps femoris*) were obtained from chick embryos ranging in age from 7 to 16 days of incubation. For electron microscopic observations, the *M. complexus* was excised at 12, 14 and 16 days of incubation and then placed in fixative. The tissues were fixed in 1 per cent O_sO_4 in Veronal acetate buffer at pH 7.2 for 90 minutes at 4°C, dehydrated in alcohol and embedded in Epon 812. The sections showing silver to light gold interference colors were cut on a Porter-Blum MT–2 ultramicrotome with glass knives, mounted on carbon-coated copper grids, and stained with uranyl acetate and Reynold's lead citrate. The sections were examined with Hitachi HW–1, HU–11 and JEM–100 U (Japan Electron Optic Laboratory Co., Ltd.) microscopes. For light microscopic observations, the *M. complexus* and *M. biceps femoris* were excised from the same embryos at 7, 8, 9, 10, 11, 12 (I, II, III, IV: 6 hr intervals), 13 (I, II, III, IV: 6 hr intervals) 14 (I, II, III, IV: 6 hr intervals), 15 (I, II, III, IV: 6 hr intervals) and 16 days of incubation. The tissues were fixed in Bouin's fluid at room temperature. The sections were stained with the PAS-hematoxylin staining method. The cell diameters of the primary and successive generation myotubes in those developmental stages were calculated by micrometer in 50 myotubes randomly selected from one slide.

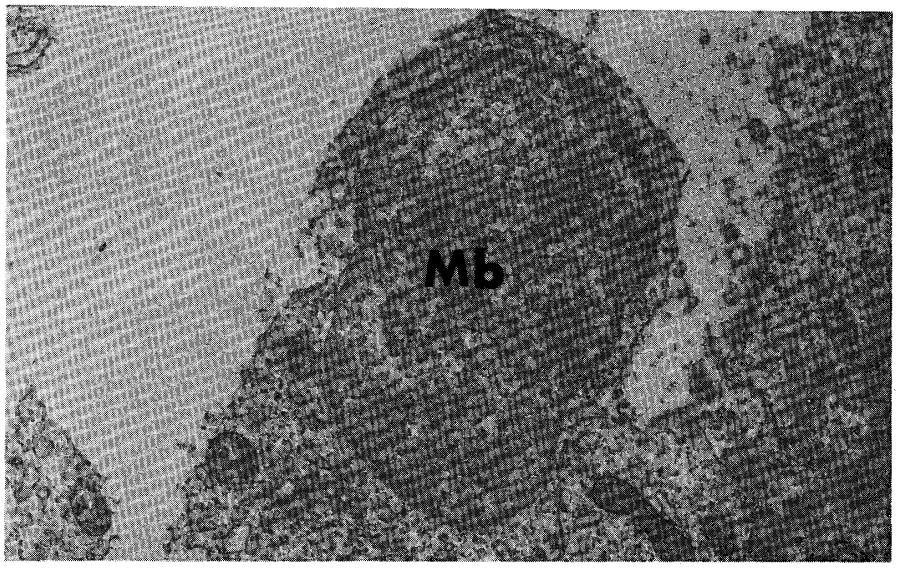

FIG. 1. Myoblasts (Mb) closed laterlay to myotube are spindle-shaped cell, and have large nucleus containing highly dispersed chromatin and Golgi complex at the perinuclear portion. ×4,600

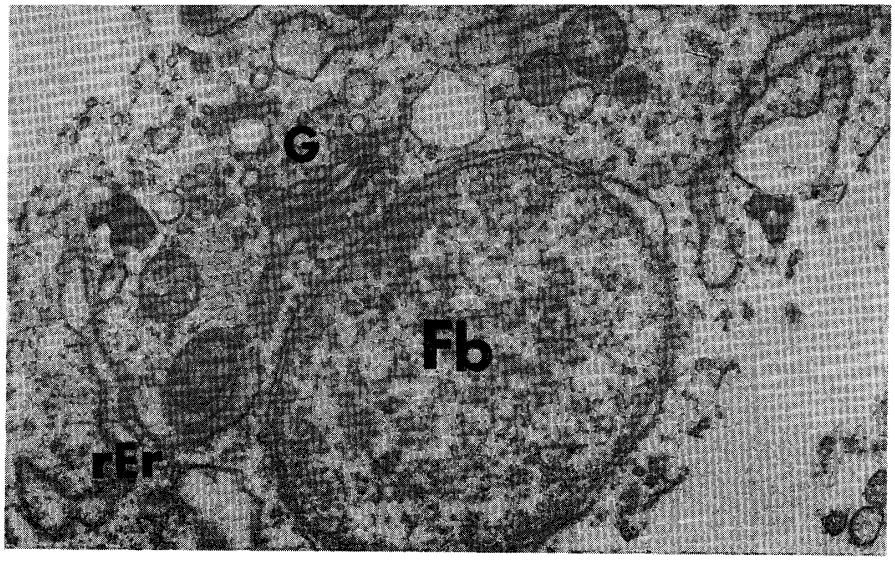

FIG. 2. Fibroblasts (Fb) are large flattened cell having well-developed rough surfaced endoplasmic reticulum (rEr) and Golgi complex (G). ×4,600

Results

1. *Ultrastructural Aspects of Myogenesis in the M. complexus*

At 12 days of incubation, the mesenchymal cells in the *M. complexus* tissues had already divided into three types of cells which could be distinguished by their cellular shape and arrangement, special disposition and subsequent behavior. The myoblasts, which were close to the myotube, were generally small spindle-shaped cells with a large nucleus containing a highly dispersed chromatin and prominent nucleoli. A small Golgi complex is present in the perinuclear portion. The distinct features of the embryonic myoblasts are many free ribosomes and a small

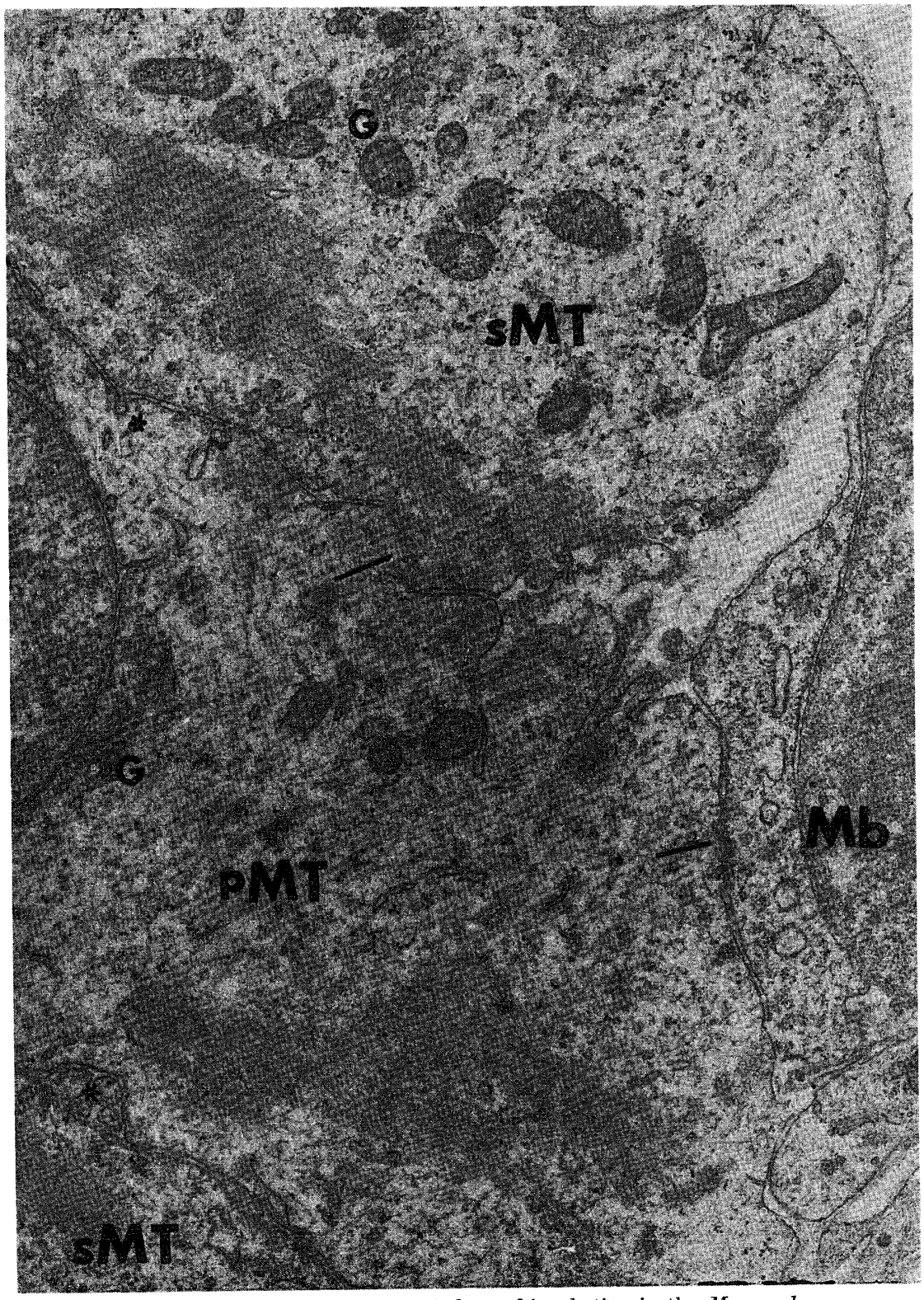

FIG. 3. Longitudinal section at 12 days of incubation in the *M. complexus*. The successive generation myotube (sMT) runs parallel to the long axis of primary myotube (pMT) and extends the tip process of cytoplasm towards myoblast (Mb). Local invaginations (*) and diffuse lines (arrow) of plasma membrane junction can be seen. ×4,600

amount of endoplasmic reticulum in cytoplasm (Fig. 1). In contrast, fibroblasts are large flattened cells having well-developed rough endoplasmic reticulum and complicated tubular structures of the Golgi complex (Fig. 2). Myotubes are divided into two groups; the primary and successive generation myotubes. The former myotubes have central large nuclei, considerable accumulation of myofibrils at the periphery of the cells, and scattered immature myofilaments throughout the cytoplasm. The latters generally surround the primary myotube, focally connect to the myotube plasma membranes by close junctions, and have immature myofibrils impartially dispersed in cytoplasm. These myotubes and myoblasts consist of a group of muscle cells which are enveloped by the same basement membrane outside of the plasma membrane. Each group of muscle cells is separated from the others by a large extracellular space (Fig. 6).

Observations of longitudinal sections show that the successive generation myotubes run parrallel to the long axis of a given primary myotube. There is some contact with the plasma membrane of the myoblast at the fuzzy lines, while others extend the tip process of the cytoplasm toward the myoblast (Figs. 3, 4). T-tubules, forming in connection with the myofibrils and having distinct Z-lines, make contact with the small vesicles formed partly in junctioned plasma membrane.

Figure 4 depicts the fusion process of the myoblast and the myotube. Their plasma membrane appears to be partly discontinuous and to form many small vesicles. Generally, the surface of the myotubes on which myoblasts and other myotubes have not yet made contact display a fold-like plasma membrane. Myoblasts are frequently insinuated to varying depths, into the interspace between adjacent myotubes by pseudopodial growth and separate them (Fig. 5, Fig. 9). Local invaginations and diffuse lines of plasma membranes can be seen in the transverse sections (Figs. 7, 8).

At 14 days of incubation numerous striated myofibrils occupy the central part of the cell in a very regular manner as seen in adult striated muscle fibers (Fig. 9). Most of the nuclei, nevertheless, are still situated centrally and muscle cells do not separate from each other, owing to the myotube membrane coalescence. As seen at the upperleft of the photograph, a myoblast insinuates into the interspace between adjacent myotubes by the pseudopodial process in the same manner as shown in Figure 5 (Fig. 9).

Myotubes in 16 days of incubation have completely mature myofibrils, other sarcomplasmic components and nuclei which space themselves in a regular manner and come to lie immediately under the sarcoplasmic membrane. Each myotube is separated by a large extracellular space in which embryonic fibroblasts appeared frequently (Fig. 10).

2. *Comparative Study of Developmental Process in the M. complexus and the M. biceps femoris by the Use of Light Microscope*

At 12(I) days of incubation, the primary myotubes are rapidly forming by the

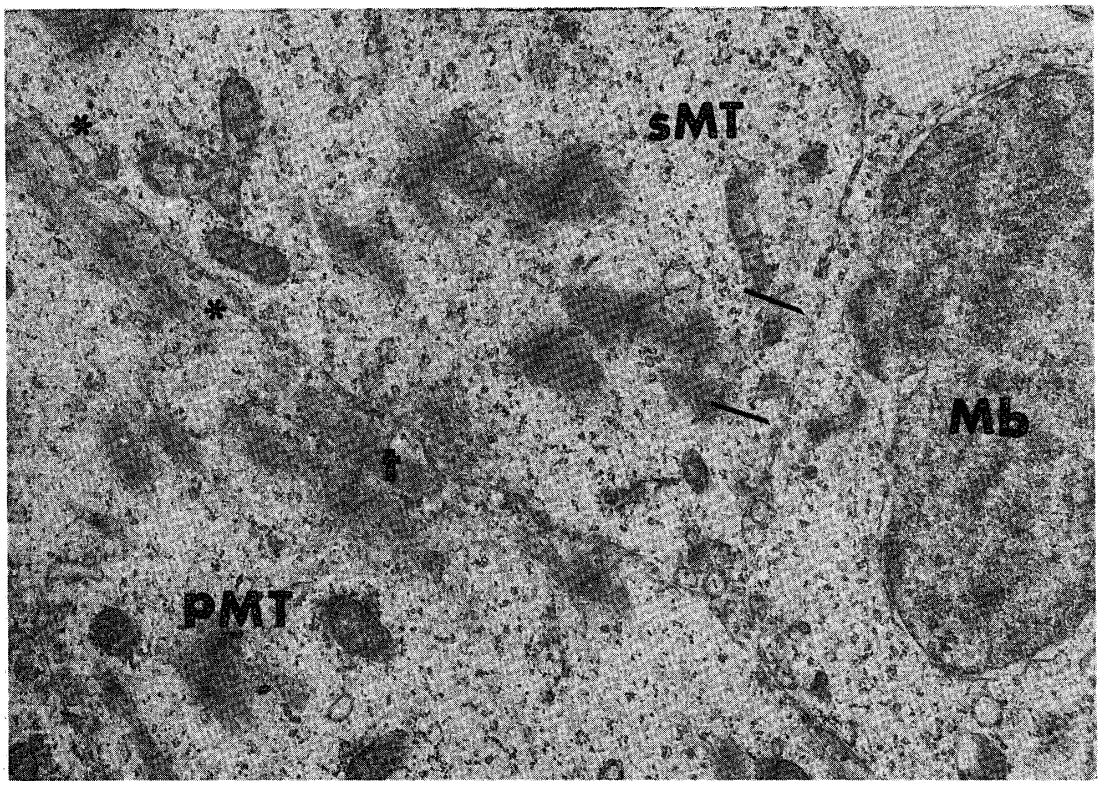

FIG. 4. Longitudinal section at 12 days of incubation in the *M. complexus*. The successive generation myotube (sMT) runs parallel to the long axis of the primary myotube (pMT) and fuses with myoblast (Mb). The fusion process of myoblast and the successive generation myotube is described by arrow. T-tubules (t) are formed in connection with myofibrils already distinguished Z-lines. ×5,000

addition of myoblasts proliferated by mitotic division. Both muscles are similar in this respect (Fig. 11. a, e).

At 12(II) days of incubation, several myotubes are formed into a group in many muscle cell clusters in which some myoblasts are close to myotubes or insinuated into the interspace between adjacent myotubes. These clusters are composed of myoblasts, the successive generation myotubes and the primary myotube which is generally centrally situated. By the use of PAS-hematoxylin staining method, we see that the myoblasts have a large nucleus and a more basophilic, myofibril-rich cytoplasm than the primary myotubes. Though both muscle tissues in this stage show little disparity in the diameter of the primary myotubes, the successive generation myotubes in the *M. complexus* are quite different from those in the *M. biceps femoris* (Fig. 11. b, f).

At 13(IV) days of incubation, the diameters of the successive generation myotubes which have separated from the primary myotube show a remarkable increase. The diameters of the primary myotubes of both muscle tissues, nevertheless, are still nearly equal (Fig. 11. c, q).

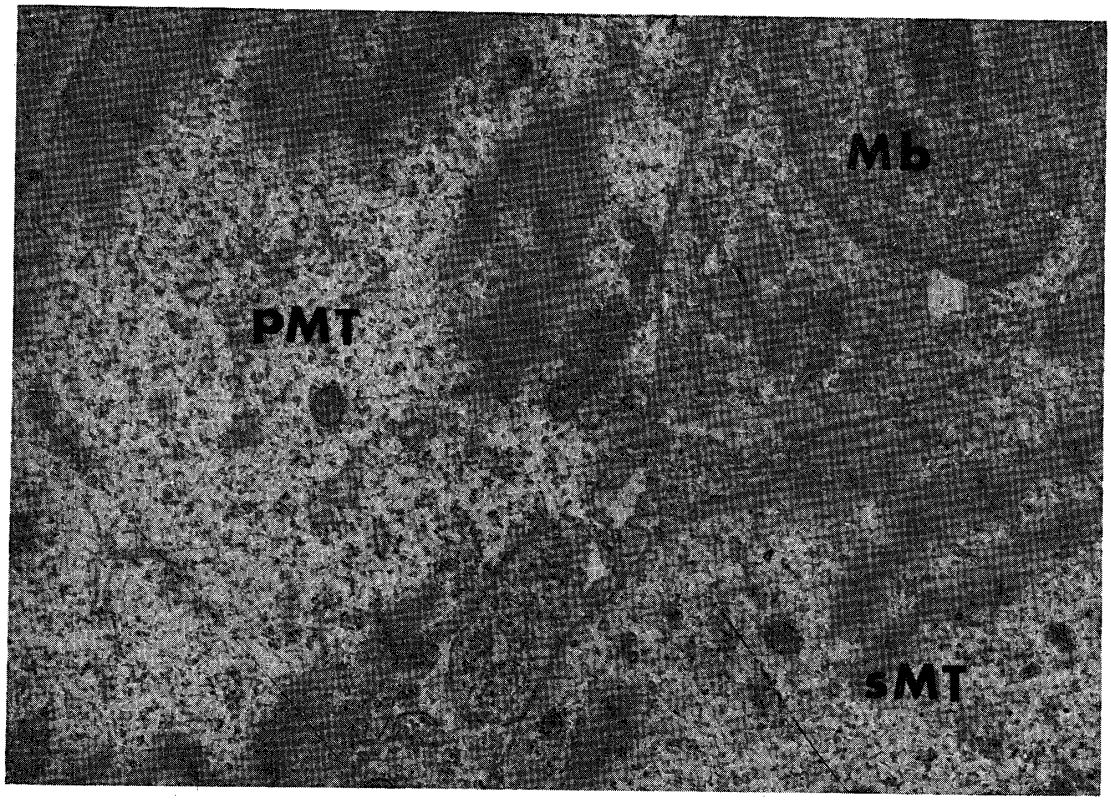

FIG. 5. Longitudinal section at 12 days of incubation in the *M. complexus*. Myoblast (Mb) is insinuated to varying depths into the interspace between adjacent primary myotube (pMT) and the successive generation myotube (sMT) by pseudopodial growth and cleaves the myotube apart. ×5,000

At 14(IV) days of incubation, the diameters of the muscle cells in the *M. complexus* are several times as large as those in the *M. biceps femoris*. The diameters continue to increase gradually until hatching (Fig. 11. d, h).

The values of the diameters of the primary myotubes and the successive generation myotubes were plotted in histogram form (Fig. 12). In both muscle tissues during various developmental stages, the primary myotubes do not show any differences in diameter. The successive generation myotubes increase gradually in cell diameter after 11 days of incubation in both the *M. complexus* and the *M. biceps femoris*. The successive myotubes in the *M. complexus* after 12(IV) days of incubation show a greater increase in diameter than those in the *M. biceps femoris*. The difference in the diameter after this stage was significant. In light microscopic observations, the primary and the successive generation myotubes in the *M. complexus* are clearly distinguishable until 13(IV) days of incubation. In the *M. biceps femoris*, however, these myotubes are distinguishable until 14(II) days of incubation.

Discussion

Some investigators have observed mitotic forms in adult muscle cells, though it is recently not accepted that muscle cell nuclei proliferate by amitosis (21, 22, 23).

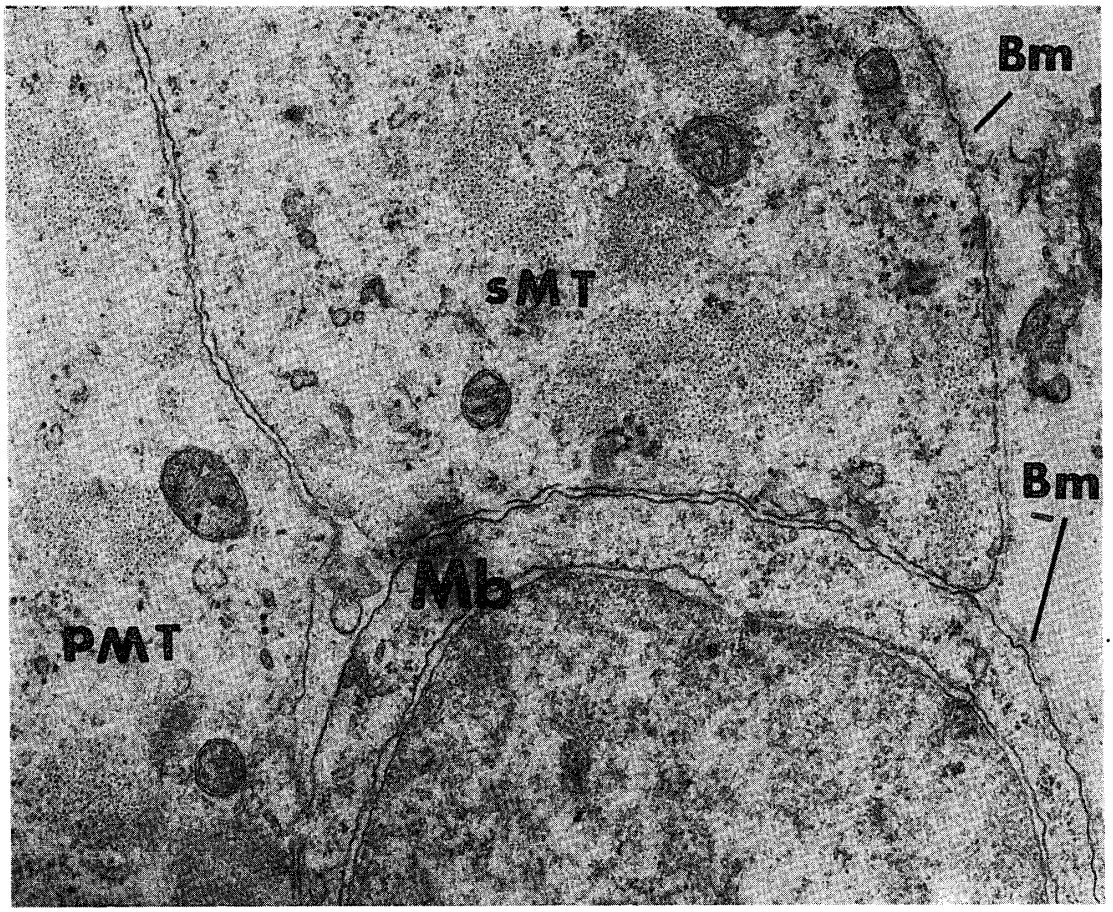

FIG. 6. Transverse section at 12 days of incubation in the *M. complexus*. The primary myotube (pMT), the successive generation myotube (sMT) and myoblast (Mb) compose in muscle cell cluster. They are enveloped by the same basement membrane (Bm) outside of plasma membrane. × 8,000

This seems to be due to the nucleic division of satellite cells, intimately associated with the muscle fiber and wedged between the fiber plasma membrane and the basement membrane (24). The above data together with that of some other authors (25, 26, 27) suggest that the mitotic divisions found in adult muscle fibers are not due to the muscle nuclei proper.

While it has been well known that the syncytial muscle fibers arise from mononuclear cells proliferating by mitotic division, opinions have differed as to the mode of increase in the number of muscle cells. Some authors thought that the new muscle cells arise by splitting or even by budding from already differentiated striated muscle cells (4, 6, 28, 29). Others have supported the theory that the myotubes are covered with many statellite cells proliferating by mitotic divisions and that this procedure plays an important role in the developmental growth of the successive generation myotubes (2, 3, 5). Pogoeff and Murray (30) studied muscle tissue culture and observed amitotic divisions of muscle nuclei, and

FIG. 7.

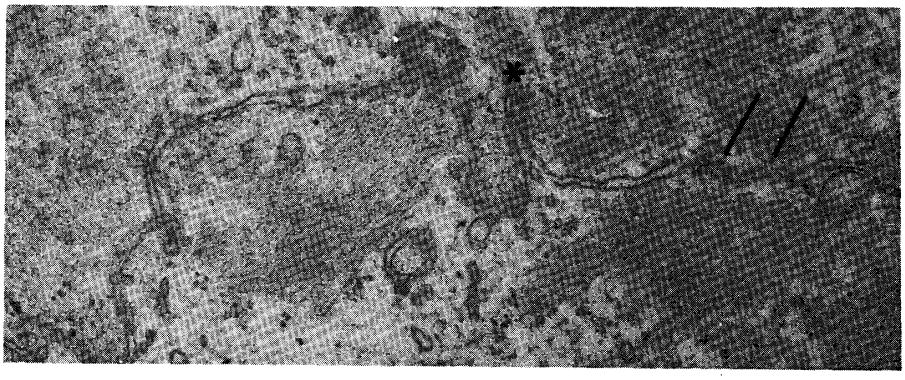

FIG. 8.

FIG. 7, FIG. 8. Higher magnification of a cross section (upper) and a longitudinal section (below) at 12 days of incubation in the *M. complexus*. The specialized membrane attachments between neighboring myotubes are local invaginations (*) diffuse lines (arrow) focal discontinuity of plasma membrane. ×21,500

accepted the former point of view.

At 12 days of incubation, myoblasts can be recognized and be distinghished from fibroblasts on the basis of their position and fine structure. Myoblasts are spindle shaped cells and have a large nucleus and have many free ribosomal granules in the cytoplasm. Fibroblasts are flat shaped cells and have a well-developed rough endoplasmic reticulum and Golgi complex. The above data are agreement with previous studies (17, 31, 32, 33, 34, 35, 36, 37). Chick muscle in this stage has clusters of muscle cells which at various stages of development were separated from other clusters by a large extracellular space. In this respect Fischman (36) has given a detailed description of the clusters of muscle cells in leg muscle of chick embryos and has suggested that all the successive generation myotubes within each cluster are destined to lateraly fuse with the primary myotube thus forming one large multinucleated myotube. The present author focused especially on the specialized membrane attachment found between neighboring myotubes observed, electron microscopically, in longitudinal sections. This phase of the fusion process between neighboring muscle cells is depicted as

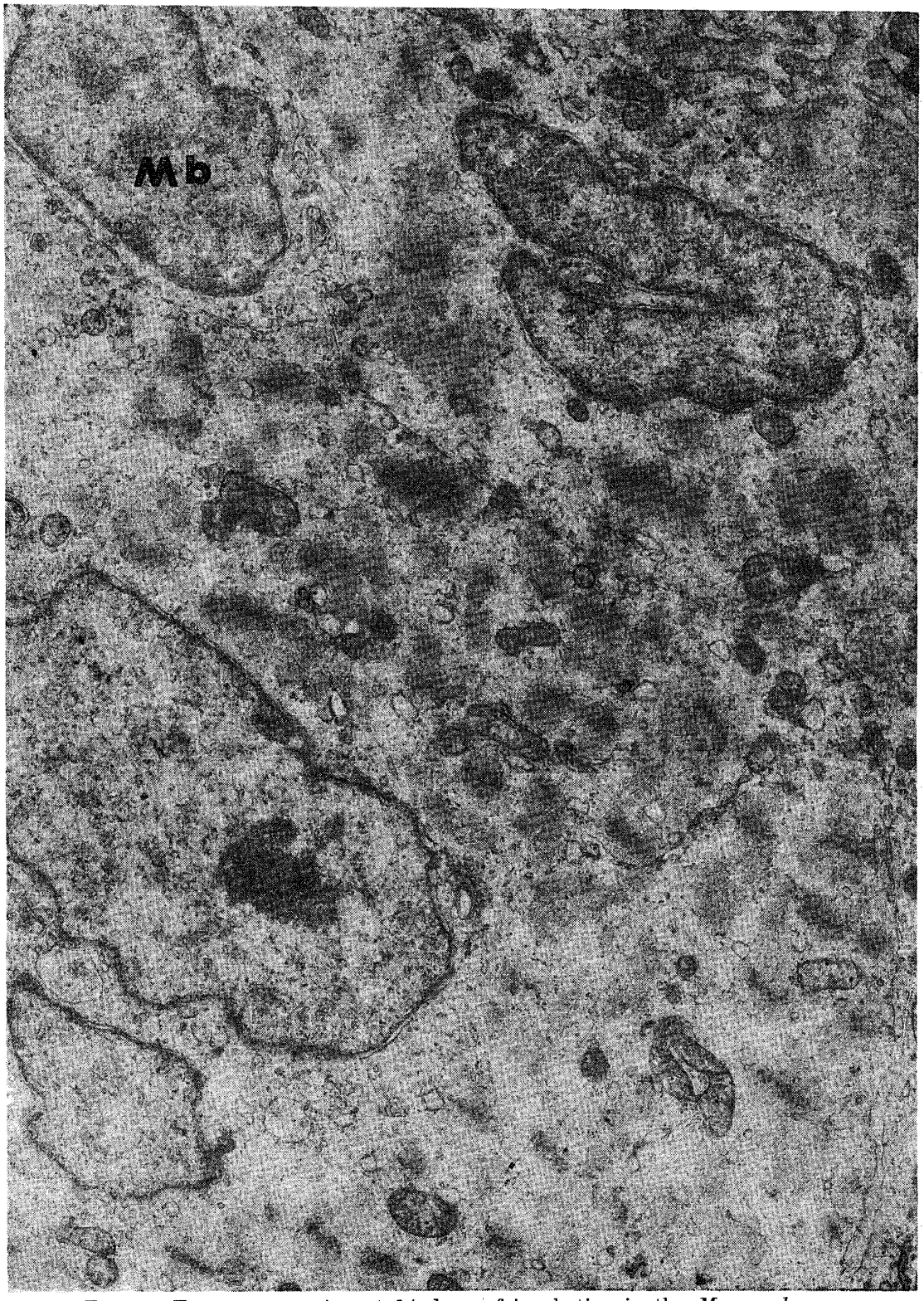

FIG. 9. Transverse section at 14 days of incubation in the *M. complexus*. Very numerous striated myofibrils come to occupy the sarcoplasm in a very reguler manner, but most nuclei are still situated centrally. Myoblast (Mb) insinuates into the interspace between adjacent myotubes by pseudopodial process. ×2,000

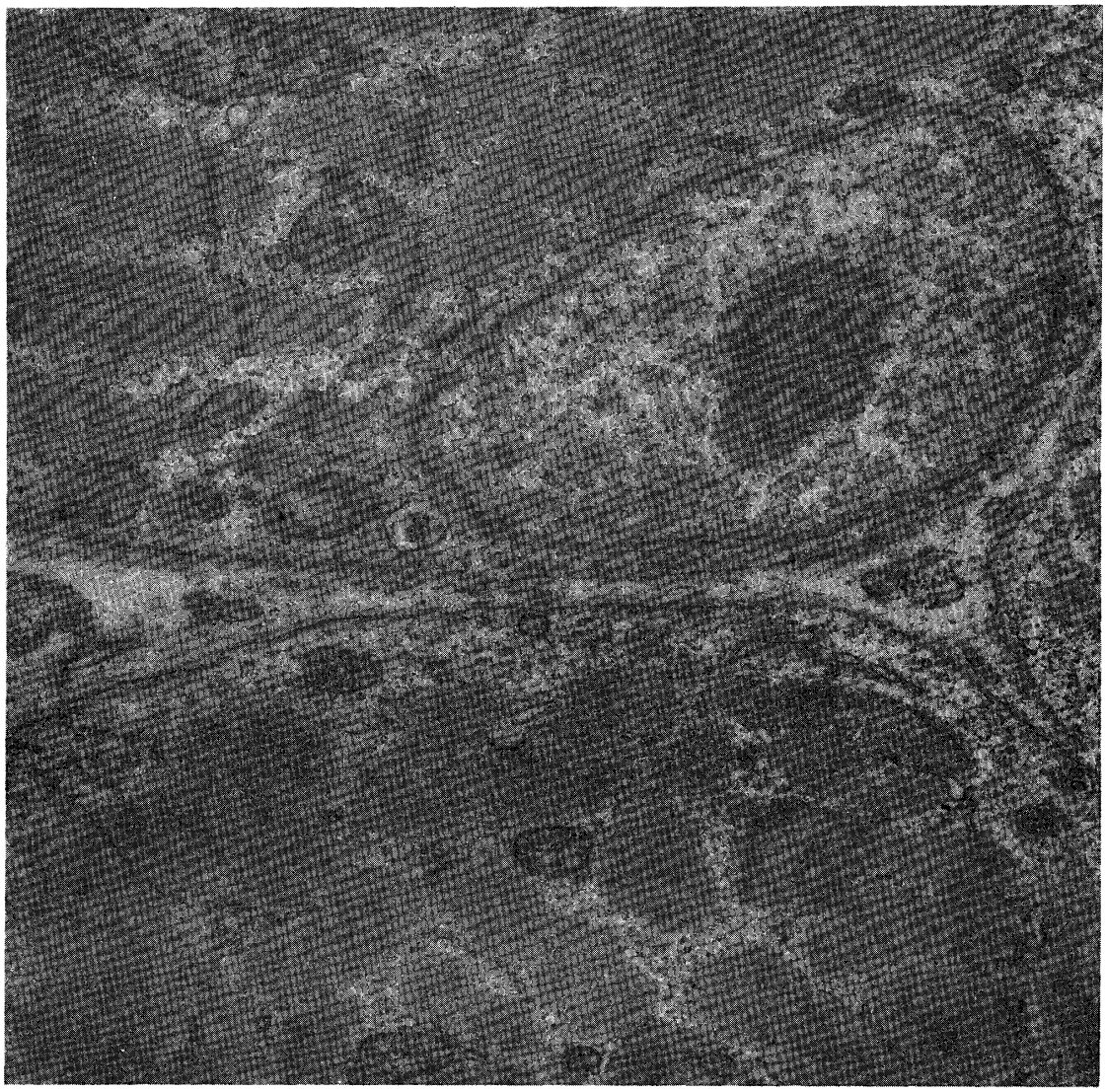

FIG. 10. Transverse section at 16 days of incubation in the *M. complexus*. Myotubes have completely mature myofibrils, other sarcoplasmic components, and nuclei come to lie under the sarcoplasmic membrane. Each myotubes are separeted by an extracellular space. ×5,000

the fuzzy lines in low magnification and as the numerous vesicles bordering the juncture in high mangification as illustrated in Figure 4.

It is a fusion-like process between neighboring myoblast and the successive generation myotube along the wall of the primary myotube. However, it cannot be determined whether the two differentiating myotubes have undergone lateral fusion or whether these myotubes are destined to separate from each other. Hay (31), Fischman (36) and Kelly and Zacks (37) gave their impressions of these specialized membrane attachments with words such as "focal discontinuous, diffusion and invaginations" found between neighboring myotubes in transverse sections. The views of Figure 3, Figure 4, and Figure 8 observed in longitudinal sections also demonstrate the conclusion of these previous workers. The fact that

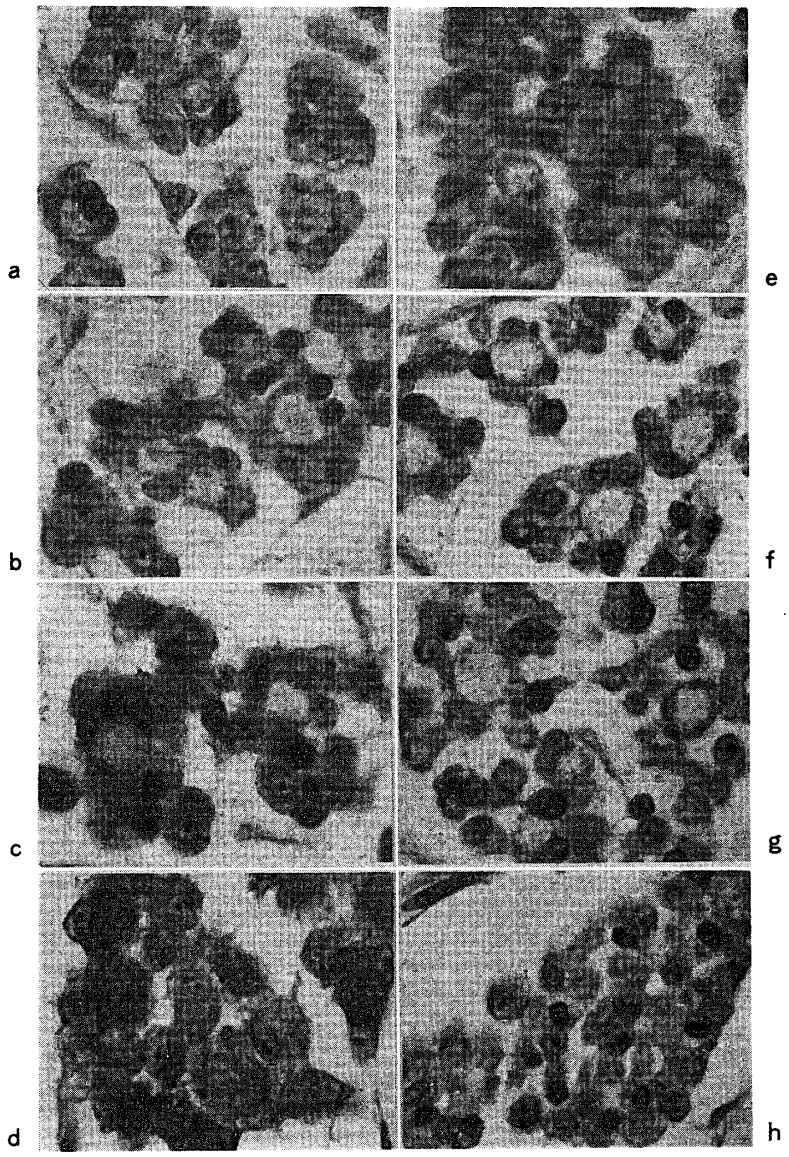

FIG. 11 A comparison of developmental process in the *M. complexus* (a, b, c, d) and the *M. biceps femoris* (e, f, g, h). a, e: 12 (I) days of incubation. b, f: 12 (III) days of incubation. c, g: 13 (IV) days of incubation. d, h: 14 (IV) days of incubation. PAS-Hematoxylin staining method. ×1,260

these fusion-like process are encountered only in limited portions and never extend over the full length of myotube in longitudinal sections clearly suggests that each myotube bordered by these obscure membranes is functionally independent of the other. Betz et al (38) claimed that the myoblast in vitro join the side of myotube and spread it out and that the delineation between the two cytoplasms, after a long time, disappears and the lump is smoothed out. Kelly and Zacks (37) reported recently that in the rat intercostal muscle the myoblast-like cell occupies a depression in the wall of the large myotube and protrudes pseudopodia along the expanded tubules farther into the myotube substance so that the cell has fused into the large myotube substance.

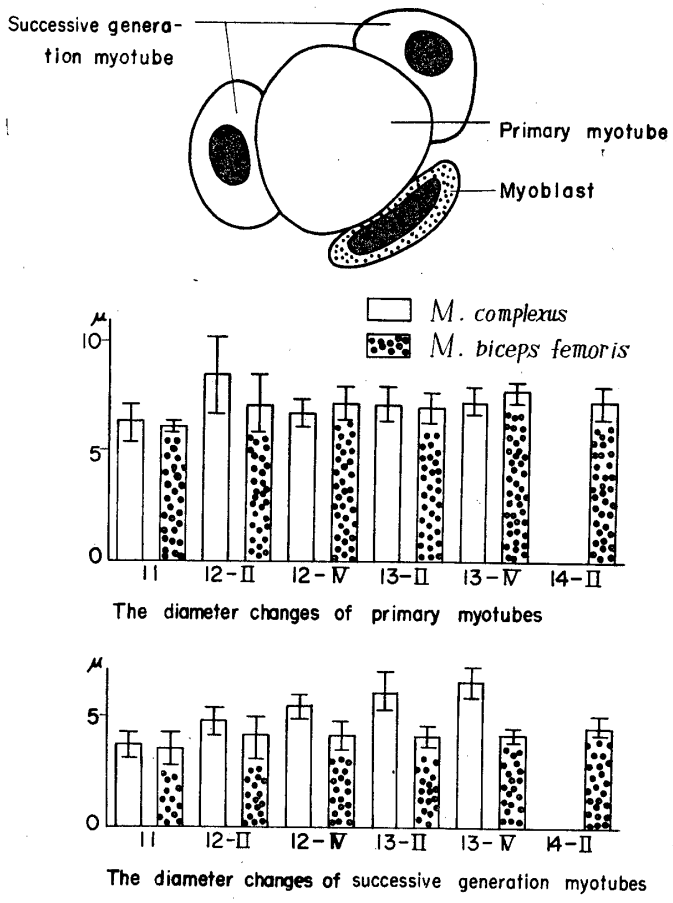

FIG. 12. The histogram of the diameter changes of the primary myotubes and the successive generation myotubes in the *M. complexus and the M. biceps femoris* at various developmental stages.

In the present study, however, these lateral fusion process between myotube and myoblast situated along the walls of the myotube was not observed. The insinuation of myoblast into the interspace between adjacent myotubes is noted in this study. It might be envisaged that the hypothesis of lateral fusion was supported more strongly by in vitro works (13, 14, 15) than in vivo works. This indicates that the muscle cells in vivo and in vitro behave somewhat differently as far as the plane of fusion is concerned.

Embryonic muscle cells of the *M. complexus* show a heavy hypertorphy at later stages of incubation in comparison with other embryonic muscle tissues (39). If muscle cells arise from lateral fusion of myotubes as advocated by Fischman (36), the primary myotubes at a certain developmental stage increase in cell diameter more than in other embryonic muscle tissues. The present study revealed that there is little difference in the diameter of the primary myotubes, but a wide difference in the diameter of the successive generation myotubes during the developmental process after 12 days of incubation between the *M. complexus* and the *M. biceps femoris* selected as the other common muscle tissue. It seemed, therefore,

that the developmental growth of muscle cells is not due to the growth of the primary myotube, but mainly to the growth rate of the successive generation myotubes, and that the mode of increase in the number of muscle cells is dependent on the separation of the new generation myotubes rather than the lateral fusion process between neighboring myotubes.

From these results of the present study, the following problems of cellular behavior may be pointed out: (1) What causes the surface of the plasma membrane of the primary myotube to form the successive generation myotubes? (2) Why the successive generation myotubes need the connecting plasma membrane with the primary myotube to increase the number of cells? (3) What controls the formation of the successive generation myotube differentiating on the overall surface of the primary myotube?

Kelly and Zacks (37, 40) recently proposed that the new generations of cells possibly differentiate along the walls of large myotubes in order to obtain support for growth, and they described later that the primitive neuro-muscular junctions were firstly present on the primary myotube, while the new generation myotubes had axonal contacts but no definitive membrane thickning to indicate endoplate formation. Shimada et al. (41) reported that nerve fibers run along the myotubes which are formed in the mixed tissue culture of embryonic spinal coad and skeletal muscle tissue. It may be concluded that there is a control system through the plasma membrane of the primary myotube, a wide difference in the affinity of myoblasts to the surface membrane of myotubes, neural control and blood supply of developmental growth. A future study along this line is planned.

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