FINE STRUCTURE OF SMOOTH MUSCLE CELLS GROWN IN TISSUE CULTURE

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ABSTRACT

The fine structure of smooth muscle cells of the embryo chicken gizzard cultured in monolayer was studied by phase-contrast optics and electron microscopy. The smooth muscle cells were irregular in shape, but tended to be elongate. The nucleus usually contained prominent nucleoli and was large in relation to the cell body. When fixed with glutaraldehyde, three different types of filaments were noted in the cytoplasm: thick (150–250 A in diameter) and thin (30–80 A in diameter) myofilaments, many of which were arranged in small bundles throughout the cytoplasm and which were usually associated with dark bodies; and filaments with a diameter of 80–110 A which were randomly orientated and are not regarded as myofilaments. Some of the aggregated ribosomes were helically arranged. Mitochondria, Golgi apparatus, and dilated rough endoplasmic reticulum were prominent. In contrast to in vivo muscle cells, micropinocytotic vesicles along the cell membrane were rare and dense areas were usually confined to cell membrane infoldings. These cells are compared to in vivo embryonic smooth muscle and adult muscle after treatment with estrogen. Monolayers of cultured smooth muscle will be of particular value in relating ultrastructural features to functional observations on the same cells.

INTRODUCTION

Ultrastructural analysis of cultured striated muscle has played an important part in investigations of the morphogenesis of myofilament formation (1, 9, 10, 16) and the origin of the transverse tubular or T-system (15, 22).

Although smooth muscles have been grown in tissue culture and studied with the light microscope since as early as 1911 (29), no reports on their ultrastructure have been found. In the present study, both phase-contrast microscopy and electron microscopy have been used to study cultured smooth muscle cells of chicken gizzard. Their fine structure is compared with that of adult (2) and embryonic smooth muscle (3, 54).

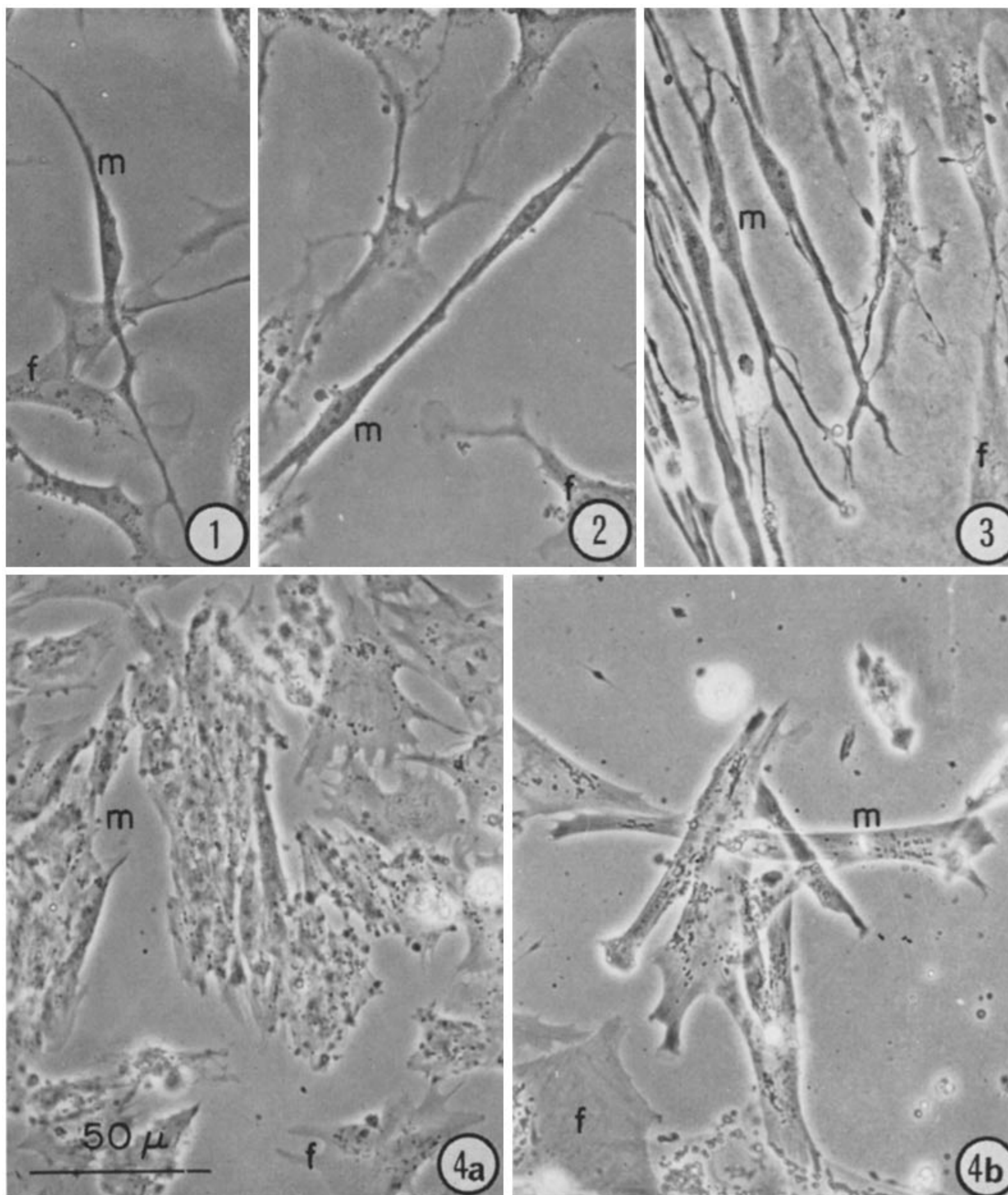
MATERIALS AND METHODS

The gizzards of 10–16-day old chick embryos were stripped of the outer serosa and inner stratum compactum layer, and the muscular tissue was dissociated into a cell suspension by using both collagenase and trypsin.[1]

In the present study, only 10- and 16-day old embryos were used, but a wider survey is at present under way.

The cells were grown on both the uncoated plastic surface of Falcon cooper dishes (Falcon Plastics, Los Angeles, Calif.; supplied by Gateway International, Los Angeles, Calif.) and on the collagen-coated (6) glass surface of Sight Tissue Chambers, Sight Instruments, Hawaiian Gardens, Calif., a modification of the original Rose chamber (41). The cultures were maintained with medium 199 (45) supplemented with 10% fetal calf serum and were examined in the living state with phase-contrast optics and time-lapse microcinematography. 3–5-day cultures were fixed for

[1] Mark, G., J. Chamley, and J. McConnell. 1970. Vertebrate smooth muscle cells in tissue culture. In preparation.



Figures 1–4 Phase-contrast photographs of living cells. Scale 50 μ (see Fig. 4 *a*) applies to all figures. *m*, smooth muscle cells; *f*, fibroblasts. × 480.

Figure 1 Muscle cell and fibroblasts. 1 day old culture of enzyme-separated cells from a 16 day embryo chick gizzard.

Figure 2 Two muscle cells in end-to-end contact. 1 day old culture of enzyme-separated cells from a 17 day embryo chick gizzard.

Figure 3 Branched contracting muscle cells. 1 week old explant culture of cells from a 17 day embryo chick gizzard.

Figure 4 *a* Muscle cells from a 1 day old culture of enzyme-separated cells from a 10 day embryo chick gizzard. These cells were actively contracting.

Figure 4 *b* Muscle cells from same culture as Fig. 4 *a* after 5 days in culture. Note the ribbon shape of the cells.

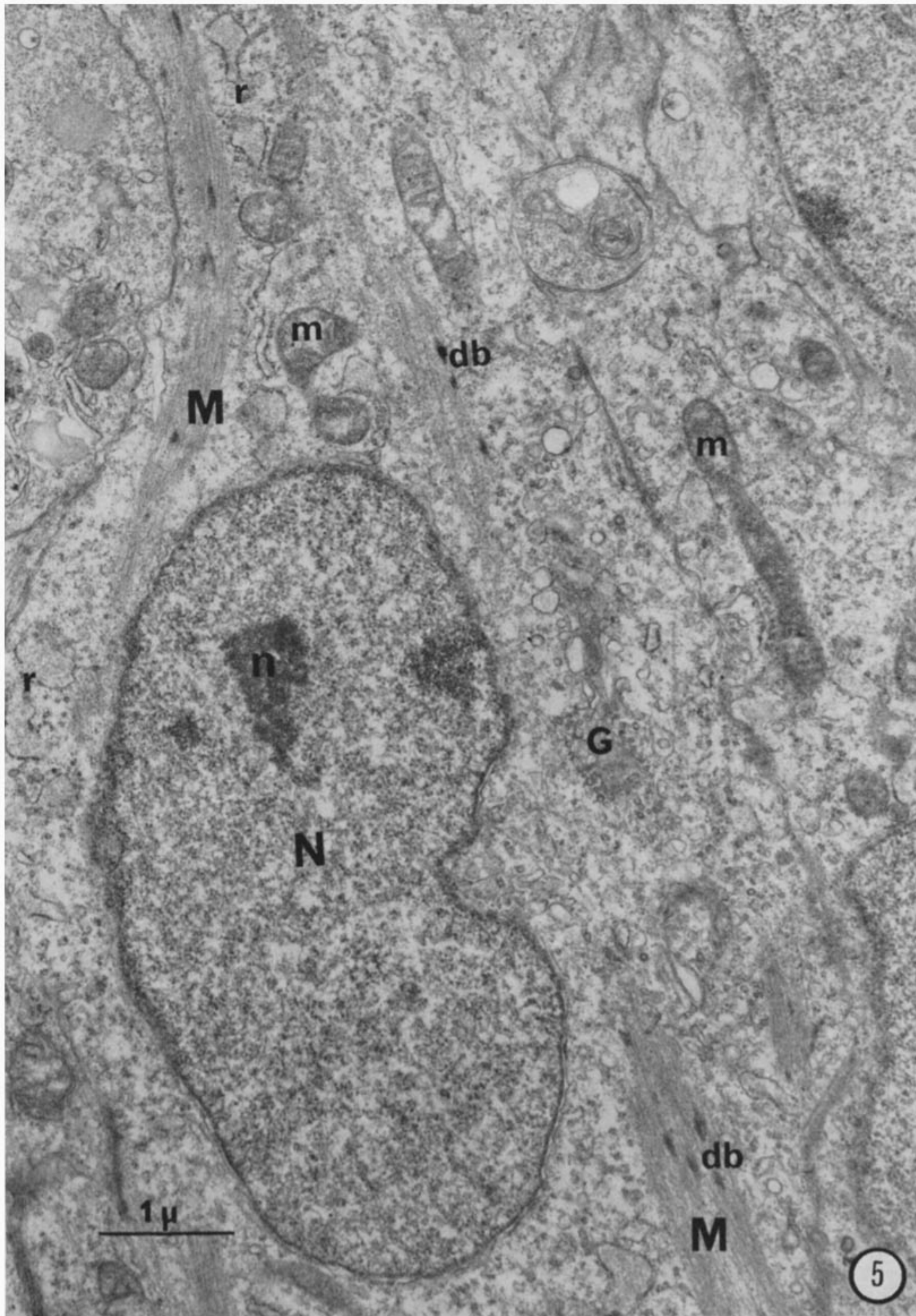


FIGURE 5 Lower power electron micrograph of cultured smooth muscle cells from 10 day old embryonic chicken gizzard 4 days in culture. Bundles of myofilaments (*M*) can be observed with associated dark bodies (*db*). The nucleus (*N*) has a smooth profile and contains a nucleolus (*n*); *m*, mitochondria; *G*, Golgi apparatus; *r*, dilated endoplasmic reticulum with free ribosomes. × 20,000.

electron microscopy in collidine-buffered 2.5% glutaraldehyde (pH 7.4) for 15 min, washed in buffer for 5 min, then postfixed in buffered 1% osmium tetroxide and in 2% uranyl acetate for 1 hr (49). After rapid dehydration through a graded series of alcohols, the cultures were embedded in Araldite (Ciba Products Co., Summit, N.J.). Infiltration with a mixture of propylene oxide and Araldite was not used, as propylene oxide dissolved the cooper dishes and was found unnecessary with a cell monolayer. After 2 days at 60°C the cultures were split off the cover slips, and muscle cells selected before fixation by phase-contrast microscopy were mounted on rods (27). Thin sections were cut on a Huxley-Cambridge ultramicrotome, stained with lead citrate (51) and examined in an Hitachi HU 11B electron microscope.

OBSERVATIONS

Phase-Contrast Microscopy

Two main morphological cell types were clearly distinguished after 1–2 days in culture. The first resembled a classical fibroblast, with an irregular shape, a large, pale nucleus, and several nucleoli of variable size (Figs. 1–4). These cells moved about the culture and changed their shape freely when seen in time-lapse cinematography. The second type of cell was identified as smooth muscle by its occasional spontaneous contractility. When taken from a 16 day embryo, these cells were characteristically bipolar, 160–260 μ long, often forked at the ends (Fig. 3), with a small oval nucleus, one to two small, regular, dense nucleoli, and a more phase-dense appearance than the fibroblasts.

Muscle cells from the 10 day gizzard were at first irregular in shape and approximately 100 μ long. After a few days in culture, they became ribbon shaped and resembled more closely the bipolar 16 day cells (Fig. 4, *a* and *b*).

Electron Microscopy

Cells in cultured gizzard were identified as smooth muscle by the presence of filaments with associated dark bodies which were similar to

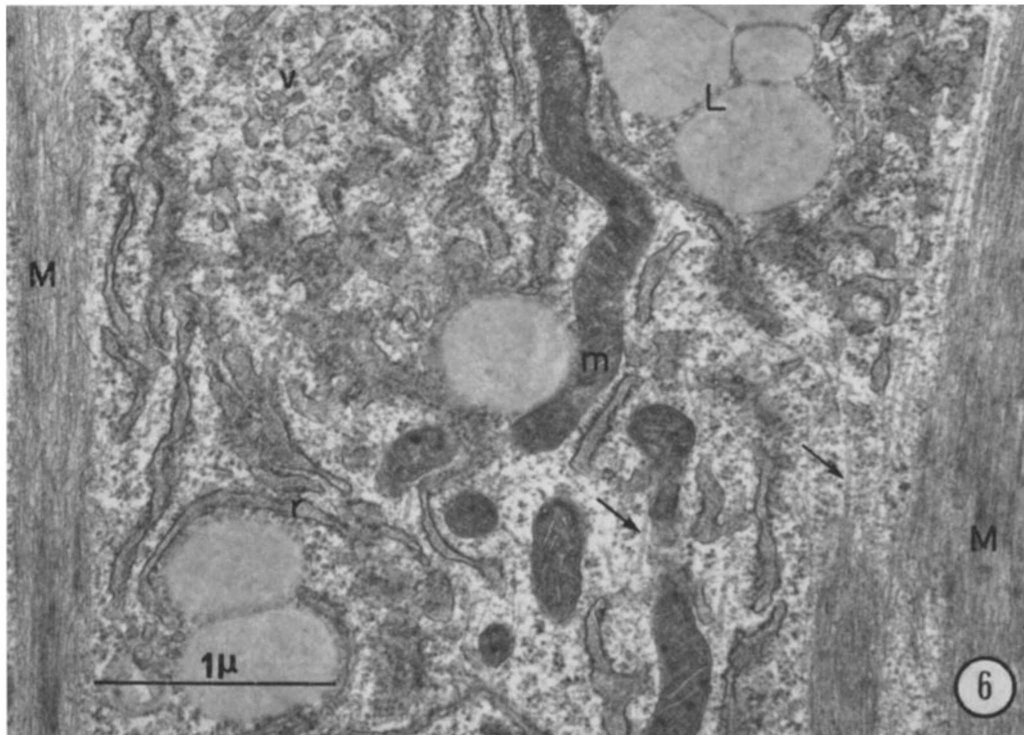


FIGURE 6 Longitudinal section through smooth muscle cell showing lipid droplets (*L*) and vesicles 500–700 A in diameter (*v*). *M*, myofilaments; *m*, mitochondria; *r*, endoplasmic reticulum; *arrows*, microtubules. × 32,000.

the myofilament–dark body arrangement seen in both embryonic and adult smooth muscle cells. However, in contrast to adult in vivo muscle, where myofilaments extend throughout the cytoplasm, the myofilaments in the cultured cells were confined to bundles in limited areas. Many cells from the 10 day old cultured gizzard showed small, randomly placed bundles (Fig. 5), whereas in the 16-day old gizzard cells the bundles were larger. The nuclei of both 10- and 16-day old gizzard cells were slightly elongated with a smooth profile and usually contained prominent nucleoli. Mitochondria, granular endoplasmic reticulum, and Golgi apparatus were particularly conspicuous and, unlike adult smooth muscle (2, 11), appeared to be distributed throughout the whole area of cytoplasm and were not restricted to the cell margin or perinuclear region (Figs. 5–7). The granular endoplasmic reticulum was often dilated with a cisternal diameter of up to 1 μ. The cisternae usually contained a moderately electron-opaque material, sometimes finely precipitated. An accumulation of lipid droplets was seen within some cells (Fig. 6). Small vesicles 500–700 A in diameter and coated vesicles 500–800 A in diameter were prominent throughout the cytoplasm (Fig. 6).

Electron-opaque particles about 200 A in diameter were very abundant and were scattered randomly throughout the cytoplasm (Fig. 7). These were of the same uniform size and appearance as ribonucleoprotein granules attached to the membrane of the endoplasmic reticulum and appeared to be free ribosomes. A number of these ribosomes were in the form of polysomes. Sometimes a number of ribosomes showed a pattern suggesting a helical arrangement, where the center-to-center distance between adjacent ribosomes was about 220 A (Fig. 8). These polysomal helices appeared to have a random orientation with respect to the myofilament bundles, and were sometimes closely associated with small filaments less than 30 A in diameter. These filaments often extended into and inter-

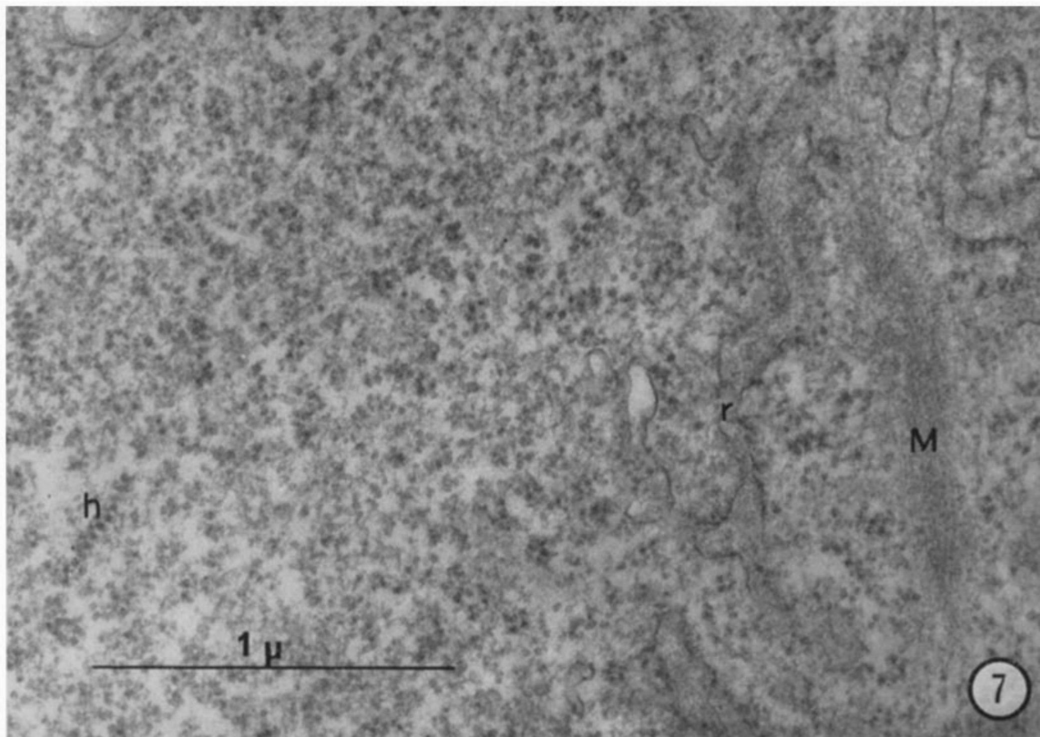


FIGURE 7 Area containing an abundance of free ribosomes and polysomes. Some of these ribosomes are in helical configurations (*h*). *r*, endoplasmic reticulum; *M*, myofilaments. × 48,000.

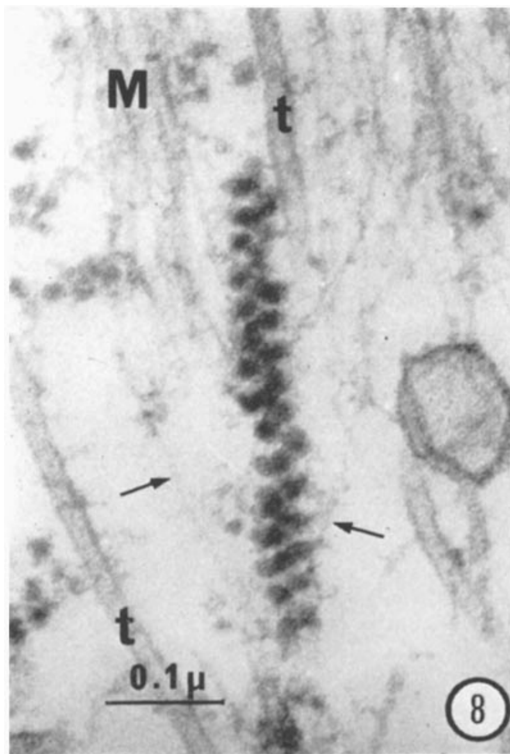


FIGURE 8 Helical arrangement of ribosomes. Small filaments (*arrows*) are seen in close association with these ribosomes. These extend into the myofilament bundle (*M*). *t*, microtuble. × 150,000.

mingled with the myofilament bundles. Particles were often seen within the myofilament bundles (Fig. 9). The particles were slightly smaller and less electron opaque than ribosomes, although they may represent glancing sections through ribosomes. However, it is possible that there is a gradation in both electron opacity and size between particles within the myofilament bundles and free ribosomes outside.

It has been suggested that both thick and thin myofilaments are present in in vivo smooth muscle (8, 25, 31, 46). A few cultured cells contained both thick (150–250 A in diameter) and thin (30–80 A in diameter) myofilaments, although the electron opacity and thickness of these thick filaments were quite variable throughout their length (Fig. 16). The majority of cells contained only the thin filaments (Figs. 5, 9).

Dark bodies were found closely associated with the myofilament bundles (Fig. 5). They appeared to be similar, although often smaller, than those of adult tissue which are reported to have a length of 700–2000 A (8). They were usually found within bundles of myofilaments, except for a few on the periphery of bundles. Under high resolution these dark bodies consisted of a dense matrix containing numerous less electron-opaque units about 70–100 A in diameter (Fig. 10).

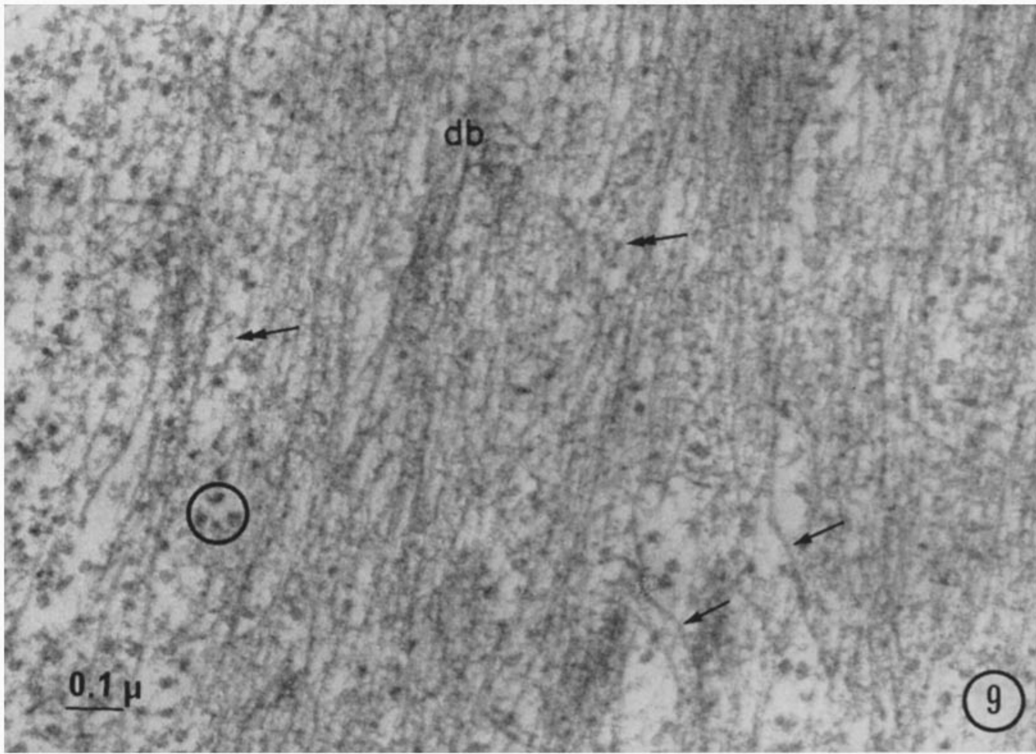
Another type of filament was found throughout the cultured cells. These filaments were 80–110 A in diameter and, unlike myofilaments, were more uniformly electron opaque. Sometimes these filaments were in the form of clusters or networks (Fig. 12). These networks were randomly orientated and the filaments appeared to be similar to the intermediate filament of cultured skeletal muscle (termed "100 A filaments" [23]). Some of these networks contained electron-opaque areas approximately 400 A in diameter and 700 A in length (Fig. 13). These areas resembled the dark bodies of the myofilament bundles. The 100 A filaments were also sometimes found in association with dark bodies between discrete bundles of myofilaments (Fig. 10). Here, the 100 A filaments appeared to pass from within the substructure of the dark body to the myofilament bundles.

Microtubules 250 A in diameter were scattered throughout the cytoplasm (Figs. 6, 8). The walls were smooth, 40 A thick, and uniform

FIGURE 9 Longitudinal section of myofilament bundle showing ribosomal-like particles. Some profiles are 250–300 A in diameter (*circle*), others are much smaller (*double headed arrows*). *db*, dark body; *arrows*, 100 A filaments. × 90,000.

FIGURE 10 Dark bodies (*db*) in association with 100 A filaments (*arrows*). The dark body appears to consist of a dense matrix containing numerous, less electron-opaque subunits. *M*, myofilaments. × 130,000.

FIGURE 11 Shows electron-opaque material (*arrow*) in association with a membranous system. *M*, myofilaments. × 160,000.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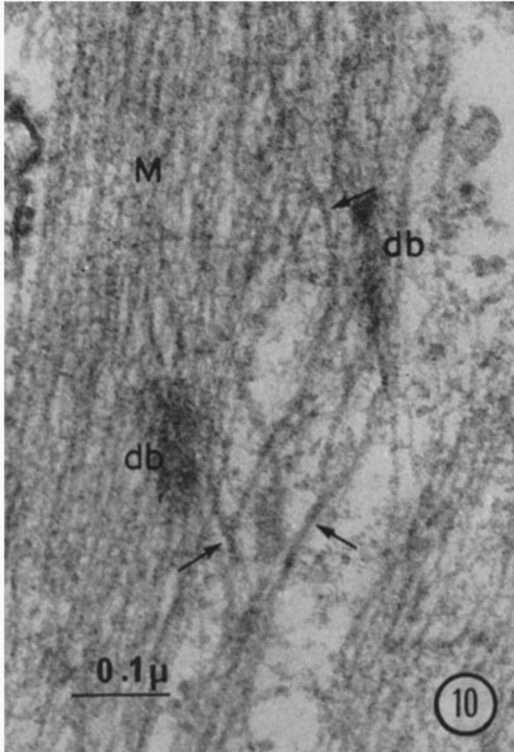
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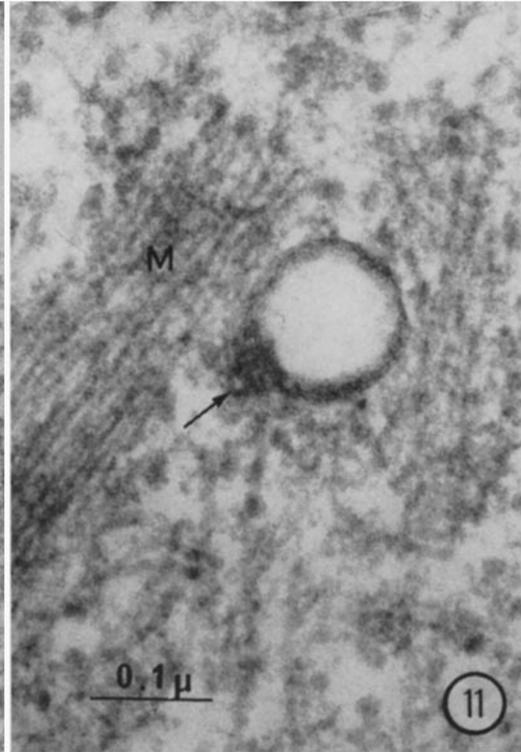
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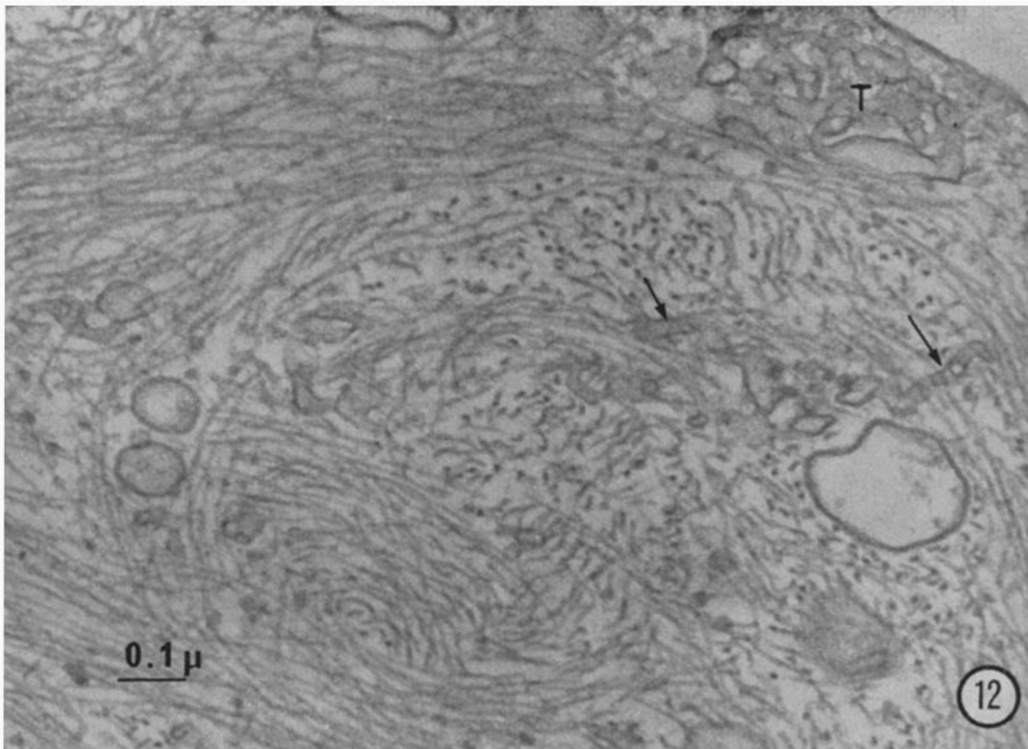
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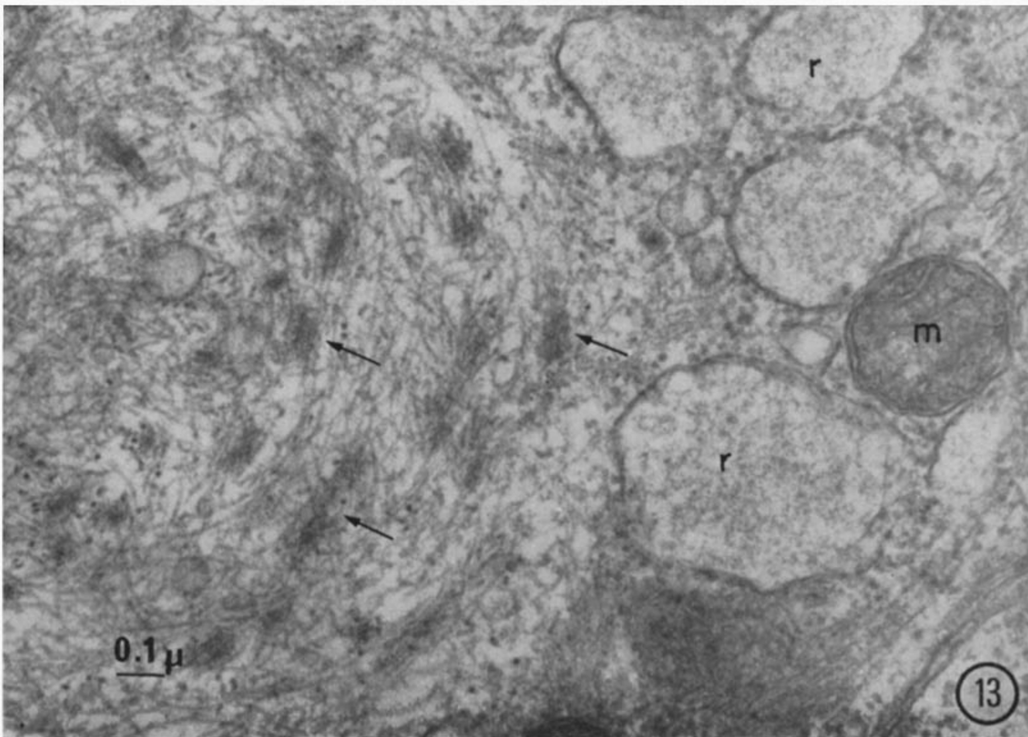
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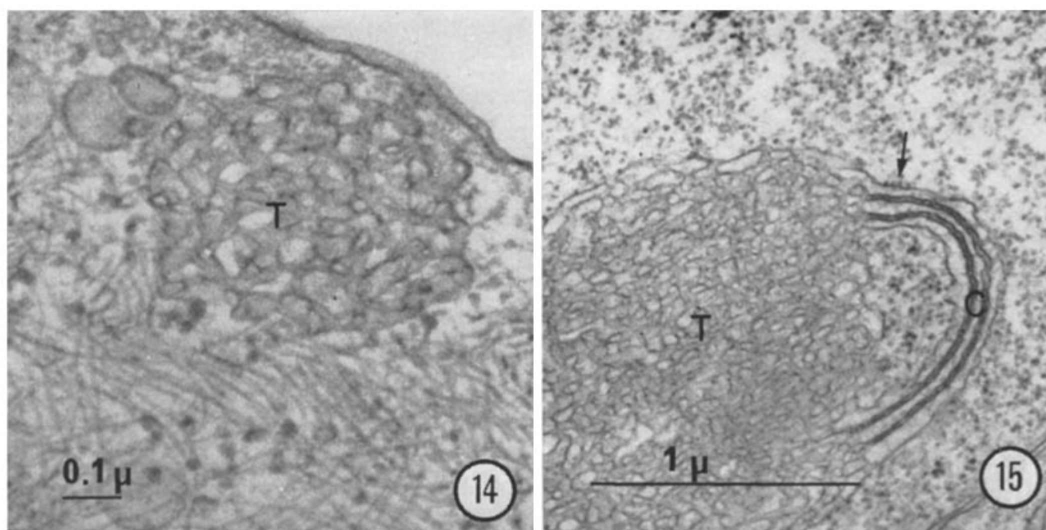


FIGURE 14 Area containing elaborate membranous network forming tubular and oblate profiles (*T*). × 75,000.

FIGURE 15 Area containing elaborate membranous network forming tubular and oblate profiles (*T*), part of which has become flattened to form cisternae (*C*). A few ribosomal particles appear to be associated with the cytoplasmic side of a cisternal membrane (*arrow*). × 36,000.

in longitudinal section. They are similar to those described in adult tissue (26).

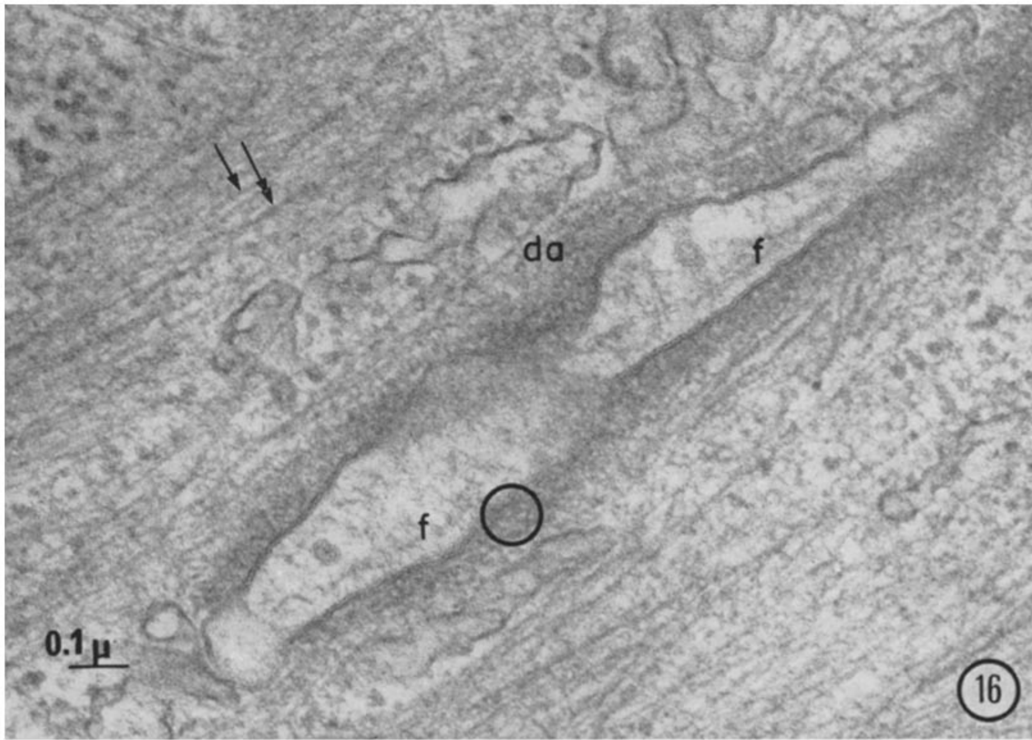
An unusual feature characteristic of some cultured smooth muscle cells, but not seen in in vivo smooth muscle, were areas containing elaborate membrane networks forming tubular (300–400 A in diameter) and oblate profiles (Figs. 12, 14, 15). These networks were usually confined to the periphery of the cell and showed some similarity to differentiating T-system tubules of skeletal muscle in tissue culture (15, 22), although in smooth muscle they had a smaller diameter and were not as well organized. Fig. 15 shows one of these tubular systems, part of which has become flattened to form cisternae. Some opposing membranes of these cisternae were closely applied to form a specialized structure, while a few ribosomal particles appeared closely related to the cytoplasmic leaflets of other cisternal membranes.

Micropinocytotic vesicles on the surface of cultured smooth muscle cells were rare and generally did not alternate with dense areas or patches as in the adult (8). These dense areas were most commonly found along distinctive infoldings of the cell membrane (Fig. 16), and extended up to 800 A into the cell. Myofilaments ran alongside the dense areas and often seemed to pass into their matrix. Some electron-opaque material was sometimes seen in association with membranous systems throughout the cell (Fig. 11).

Cultured smooth muscle cells in close contact with each other were usually separated by a gap of 200 A, increasing to 300–400 A when basement membrane substance was present. This is somewhat less than the basement membrane–filled

FIGURE 12 Clusters or networks of filaments 80–110 A in diameter found within some cultured smooth muscle cells. *T*, network of tubules; *arrows*, part of tubular system. × 78,000.

FIGURE 13 Network of filaments containing electron-opaque areas approximately 400 A by 700 A (*arrows*). These areas resemble the dark bodies of myofilament bundles. *m*, mitochondria; *r*, dilated endoplasmic reticulum. × 65,000.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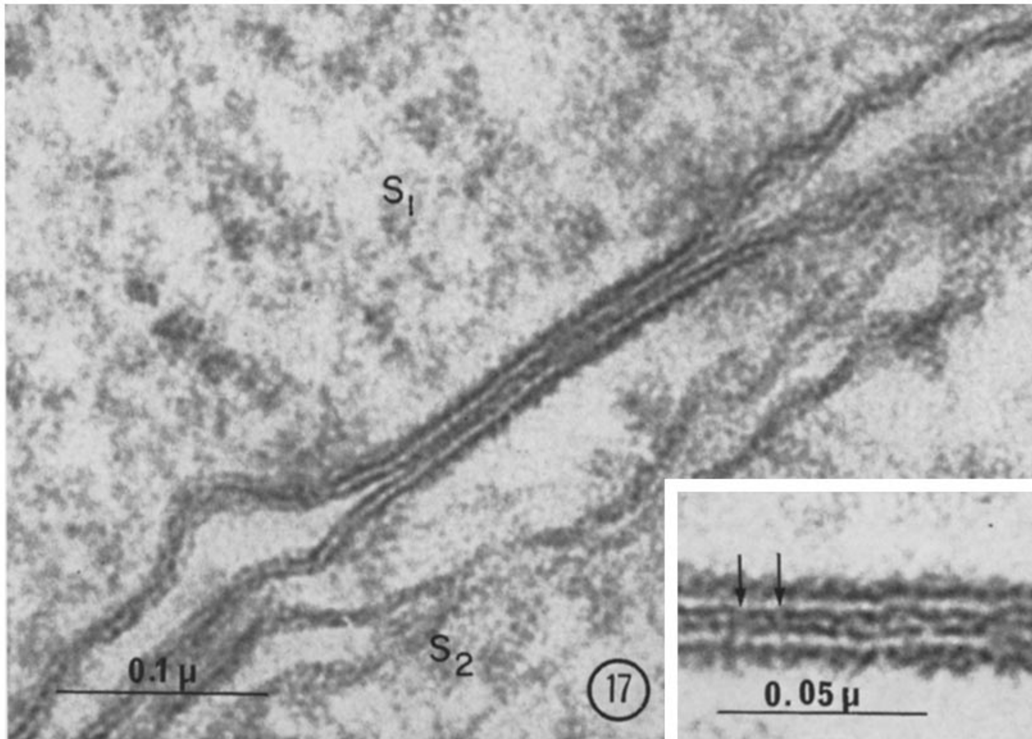
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gap of 500–800 A between most adult cells (8). Regions of close apposition forming nexuses were occasionally observed between muscle cells (Fig. 17). The outer leaflets of the membrane were separated by a gap of up to 30 A, except for a few short areas of fusion. These are, therefore, best described as "gap junctions" and have also been shown to occur in adult smooth muscle (38, 50). The inner leaflets of the membranes of these junctions were lined with electron-opaque material.

Sorokin (47) described the process of ciliogenesis in both smooth muscle and fibroblasts. Some evidence of this process was seen in cultured smooth muscle.

Membrane-bound particles about 1000 A in diameter, containing an electron-opaque core, were seen in some cultures. These particles resembled those seen in embryonic tissue (3) and were quite similar to viruses associated with Marek's disease (30).

DISCUSSION

Cultured smooth muscle shows many of the characteristic features of in vivo embryonic smooth muscle (3, 54), of smooth muscle in response to estrogen (17, 43), and of regenerating smooth muscle of anterior eye chamber transplants.[2] These features include an abundance of free ribosomes, dilated rough endoplasmic reticulum, and Golgi apparatus, all suggesting active protein synthesis.

One must be cautious in applying results from in vitro studies to in vivo studies. There may be differences in fine structure due to the artificially imposed culture milieu, and there may also be differences related to factors such as lack of innervation. However, cultured smooth muscle has the particular advantage that it can be used to study the relation of ultrastructural features to functional observations on the same cell.

A number of authors have suggested that polysomes or free ribosomes play a major role in the synthesis of myofilaments in striated muscle (4, 7, 18, 19, 52, 53). Isolated polysomes, containing 60–70 ribosomes, from 14 day old embryonic chick skeletal muscle, have been shown to synthesize myosin (20, 21). By analogy, the large aggregates of ribosomes seen in both embryonic and cultured smooth muscle may also be involved in myosin synthesis. Leeson and Leeson (28) describe the occurrence of polysomes and free ribosomes in developing smooth muscle. These become less marked in later development. Yamauchi and Burnstock (54) noted a similar transient appearance of ribosomal rosettes and correlated this with the first appearance of coarse myofilaments (60 A in diameter) and dark bodies, although fine filaments (less than 30 A in diameter) were established earlier. In these studies no definite evidence of structural connections between polysomes and myofilaments was shown.

Although myosin is present in smooth muscle (31) and follows a pattern of aggregation similar to that of myosin of striated muscle (24), the question of the in vivo form of this myosin is a subject of current debate. Kelly and Rice (25) have shown that thick filaments are present in contracted muscle and have suggested that these are myosin filaments which aggregate during contraction and disperse when the muscle is relaxed. Panner and Honig (34, 35) were unable to find these thick filaments in any stage of contraction. They have, however, shown structures morphologically similar to myosin molecules and/or dimers in both the contracted and

[2] Campbell, G. R., Y. Uehara, T. Malmfors, and G. Burnstock. 1970. Degeneration and regeneration of smooth muscle transplants in the anterior eye chamber: an ultrastructural study. *Z. Zellforsch. Mikrosk. Anat.* In Press.

FIGURE 16 Dense areas (*da*) with a well-defined substructure (*circle*) prominent along an infolding of the cell membrane. Myofilaments seem to pass into the dense-area matrix. The extracellular space of the membrane infoldings contains filamentous substance resembling basal lamina material (*f*). What appear to be thick (*double headed arrow*) and thin (*arrow*) myofilaments can be distinguished. × 80,000.

FIGURE 17 Gap junction between two cultured smooth muscle cells (S_1, S_2). *Inset* shows high magnification of part of this junction. A gap of up to about 30 A can be seen between the outer leaflets of the unit membrane; there are a few short areas of fusion (*arrows*). The inner leaflets of the membranes are lined by an accumulation of electron-opaque material. × 280,000. *Inset*, × 560,000.

relaxed state. Only a few cultured smooth muscle cells contain thick myofilaments, the diameters of which are quite variable. This suggests: (*a*) that thick filaments are either aggregations or random superimpositions of thin filaments, (*b*) that cells within the same culture can be fixed in different stages of contraction, (*c*) that cells within the same culture can be at different stages of differentiation, as Bennett and Cobb (3) saw only thin myofilaments in the early stages of development of chicken gizzard.

Randomly orientated networks of filaments (100 A filaments) are a feature of cultured smooth muscle. Similar filament arrangements have also been seen in smooth muscle of 10 day old chick embryo gizzard, and these filaments are also present in adult tissue but in far smaller numbers.[3] Cytoplasmic filaments have also been observed in a variety of other tissues. Salazar and Totten (44) noted patches of filaments in cells of human gastric leiomyoblastomas. However, these were 60–80 A in diameter and were thought to be actin. Similar filaments have also been described in rat liver epithelial cells (5), in cells of blood-forming organs (48), and in mononuclear phagocytes (12), where their function may be cytoskeletal. Ishikawa et al. (23), although unable to determine the exact nature and function of the 100 A filaments in cultured skeletal muscle, provided evidence that they were not involved in myofibrillogenesis. The same may apply to smooth muscle. They did, however, find evidence which supported the theory of O'Brien and Thimann (33), who suggest that microtubules and filaments are alternate states of assembly of the same subunit particle. In cultured smooth muscle evidence to support this theory was not found. Nevertheless, a close relationship between 100 A filaments and structures very similar in appearance to dark bodies was evident. If these structures are dark bodies, this suggests that the filaments play a role in dark body formation and/or form part of their substructure. A number of authors have suggested that dark bodies correspond in function to the Z lines of skeletal muscle (8). The possibility therefore arises that they may be derived in a similar manner. Heuson-Stiennon (19) suggests that in embryonic rat skeletal muscle Z lines appear to form from dense bodies formed at the cell membrane. Consequently, another possibility is that dark bodies in smooth muscle form from dense areas along the membrane or even from electron-opaque material seen in association with some membranous systems.

Elaborate networks of tubules, similar to those found in cultured smooth muscle, have also been observed in denervated rat skeletal muscle (37), in plastids of some plants (32), and in the chloride cells of the fish gill (14). The functional significance of these networks reamains to be understood. However, Ishikawa (22) suggests that similar networks of tubules found in both cultured and atrophied skeletal muscle are due to abnormal T-system proliferation, and that these may be indirectly due to the absence of innervation. The same could be true of cultured smooth muscle. Further studies need to be made to understand the true function of these networks.

Myofilaments have often been reported to be attached to dense areas on the cell membrane of adult smooth muscle (26, 34, 36, 39, 40, 42). These dense areas presumably act as points of attachment upon which myofilaments can develop tension. In cultured smooth muscle, dense areas along infoldings of the cell membrane probably play a similar role, since myofilament bundles seldom extend to the cell margins but are confined to patches within the cell.

Nexuses appear to be the morphological basis of electrical interaction between individual smooth muscle cells in adult organs (8). However, they may have other or additional functions, since they have been described between fibroblasts in tissue culture (13), and between protrusions of the same smooth muscle cell in guinea pig ureter.[4] An interesting feature with respect to the nexuses found in cultured smooth muscle is that most of the cells were separated by enzyme dissociation in the culturing procedure, so that any nexuses present were likely to be newly formed.

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